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94. ISOLATION OF ADENINE-DESOXYRIBOSIDE FROM THYMUSNUCLEIC ACID

By THOMAS G. BRADY

From the Department of Biochemistry, University College, Dublin

(*Received 7 March 1941*)

In the course of deamination experiments it was found necessary to prepare a sample of adenine-desoxyriboside. This substance has already been isolated by Klein [1934; 1938]. Using his method, which, however, it was found necessary to modify considerably, the substance obtained had a very much higher sintering point and a distinctly higher melting point than described by Klein. The crystalline nucleoside isolated in this Laboratory sintered at approximately 160° and melted sharply at 187–188°.[1] The data given for adenine-desoxyriboside by Klein show that his product sintered between 120 and 125° and melted sharply at 181°.[1] Klein [1934] showed that the M.P. varied with the rate of heating; with slow heating the substance melted some degrees below 181°. The same effect was observed with the higher-melting compound described in this paper, but at no time was the M.P. below 184°.

In other properties, e.g. solubility, crystalline form and rotation, the substance is very similar to Klein's. The analytical data for C, H and N, are in good agreement with the theoretical values, and the substance gives a quantitative yield of NH_3 when deaminated with tissue extracts. It therefore seems reasonably certain that the higher-melting substance is in fact an adenine-desoxyriboside. The possibility that it is an isomeride differing from Klein's in the attachment of the sugar molecule to the aglucone cannot, however, be overlooked.

Gulland & Story [1938], using a sample of Klein's adenine-desoxyriboside, proved by absorption spectrum analysis that the sugar molecule is attached to position 9 of the purine skeleton. The point of attachment of the higher-melting substance may conceivably be at position 7. This, of course, would be of considerable interest, as it would be the first example of a nucleoside with the sugar attachment at position 7. Unfortunately no final decision can be reached on this question, as the facilities at present available in this laboratory do not allow of an absorption spectrum measurement being carried out. The alternative, considerably strengthened by observations made on thymine-desoxyriboside, that the higher-melting substance is merely a purer form of adenine-desoxyriboside seems, however, more probable. It was found, for example, that an impure sample of thymine-desoxyriboside after as many as four recrystallizations from distilled water melted 10° below the correct M.P. of this substance. One recrystallization of this same impure sample from 50% water/alcohol gave a substance which melted correctly at 185°. It appears from this, that unless a suitable solvent is used, minute amounts of impurities may adhere to these nucleosides and cause a considerable depression of M.P. In the case of adenine-

[1] All melting points recorded in this paper are uncorrected. The possibility that the divergence in the melting points may be due to differences in the thermometers used, is ruled out by the fact that the thymine-desoxyriboside isolated in this laboratory melts at exactly the same temperature as that recorded by Klein, i.e. at 185°, almost midway between the divergent melting points.

desoxyriboside it was found advantageous to use a water/acetone mixture instead of absolute alcohol. By this means, adenine-desoxyriboside invariably crystallized out on standing overnight in the refrigerator, and the long and tedious procedure adopted by Klein of allowing the nucleoside to crystallize from absolute alcohol (1–2 weeks) was found to be unnecessary. At the same time it appears that a purer product has been obtained.

The various modifications of Klein's method are fully described in the experimental section and need not be further discussed here. There is, however, one point not mentioned by Klein in any of his communications on the desoxynucleosides and nucleotides, which appears to be of considerable importance, namely, the effect of the depolymerizing enzyme 'nucleogelase'. The work of Feulgen [1935] and Schmidt & Levene [1938] on nucleogelase has proved that this enzyme breaks down polymerized *a*-thymusnucleic acid to depolymerized *b*-thymusnucleic acid. One would expect Klein's thymusnucleic acid prepared by the method of Feulgen [1914] to contain much polymerized *a*-thymusnucleic acid, and this form is not attacked by nuclease [Schmidt & Levene, 1938]. Yet Klein obtained 96% dephosphorylation in the preparation of the nucleosides. Feulgen suggests that the thymonucleinase of Klein is associated with the nucleogelase, noting that the *p*H optima of these ferments are close together, but his figure of 6·5 for the thymonucleinase is incorrect, the true value being *p*H 9·2 [Klein, 1932]. Klein and his co-workers for preparative work [Klein, 1932; 1933; 1934; 1938; Bielschowsky & Klein, 1932] selected a slightly lower *p*H (8·8–9·0) than the optimum but at this point nucleogelase, according to Feulgen himself, is inactive.

The dephosphorylation experiments carried out in this Laboratory gave consistently low values for hydrolysed phosphorus (50% of total organic P) and in consequence the yield of adenine-desoxyriboside was only 50% of that obtained by Klein. Depolymerization experiments, using Feulgen's method, but not using Merck's pancreatin, were attempted, but with little success. It is probable that the pancreatin employed contained very little nucleogelase. The quantity of nucleophosphatase was increased to threefold that used by Klein, but even then only 60% of the total organic P was hydrolysed.

Another factor influencing the dephosphorylation very considerably was the addition of Ag-ion as a deaminase inhibitor. This reduced the hydrolysis of PO_4-ion by about one-third. The average amount hydrolysed was approximately 20% of the total organic P.

Experimental

Enzyme solution. The preparation and purification of the enzyme nucleophosphatase from calves' intestines were carried out according to the direction of Klein [1938]. It was found an advantage, however, to modify slightly the initial step in the purification by using 2 volumes instead of 1 volume of acetate buffer, and addition of the glycerol extract to the acetate solution. Although this procedure increased the volume of solution to be centrifuged, it counteracted any tendency for the precipitate to come out in a colloidal form, which by the original method sometimes occurred, and was found very difficult to rectify.

Hydrolysis of thymusnucleic acid. The enzymic hydrolysis was carried out on 10 g. of thymusnucleic acid. The procedure adopted was almost identical with that of Klein [1938], except that the concentrations of enzyme and Ag used were trebled. The hydrolysed P was estimated colorimetrically [Bomskov, 1932].

Treatment of the hydrolysed thymusnucleic acid. Klein's technique was used for the precipitation of the $PO_4^{\equiv}$ and for concentrating the solution. The centri-

fuging of the cooled gel was attempted but was found to be unsatisfactory, and absolute alcohol was added until the precipitate which formed was quite powdery. To ensure complete extraction of adenine-desoxyriboside the powder was shaken three times with alcohol. The combined extracts were concentrated *in vacuo* at 40° and removal of inorganic ions (NH_4, Mg, SO_4) and neutralization, were carried out as outlined by Klein [1938]. Amorphous substances, probably of protein nature, were removed by repeated precipitation with absolute alcohol. The filtrate was concentrated to about 5 ml. Further concentration was effected by passing a filtered stream of air through a suction tube, containing the filtrate and washings in a small test tube, heated to 40° in a water bath. When all the alcohol had evaporated, acetone was added giving a gummy residue. On addition of a few drops of water, this dissolved, and on standing overnight in the refrigerator, adenine-desoxyriboside crystallized out in typical block-shaped crystals.

Most of the acetone was removed by evaporation in an air stream as described above. On standing in the refrigerator for a few hours the residue had set to a solid mass. The crystals were filtered off on a small filter, using a glass button. The crystalline mass, which was sometimes admixed with gummy material, was dissolved in water heated to 60°. A small quantity of a water-insoluble, noncrystalline material was filtered off. After a few hours in the refrigerator adenine-desoxyriboside crystallized out. Yield 120 mg., sinters 162–165° M.P. 187–188° (uncorr.).

It was recrystallized three times for analysis without alteration of M.P. $[\alpha]_D^{20°} -26{\cdot}9$ (in water). Found: C, 48·00; H, 5·42; N, 28·30; H_2O, 6·35. Calc. for $C_{10}H_{13}O_3N_5, H_2O$: C, 47·78; H, 5·22; N, 27·88; H_2O, 6·69%. (Analysis by Drs Weiler & Strauss, Oxford.)

Quantitative estimation of amino-N by the Conway technique [Conway, 1935; Conway & Cook, 1939]. 0·5 ml. of a solution containing 0·5 mg. adenine-desoxyriboside was added to 3·5 ml. phosphate buffer *p*H 6·1. To this was added 1 ml. of the enzyme solution, made by shaking 50 mg. of acetone-dried intestinal mucosa with 10 ml. water. Found amino-N, 5·08; 5·35. Calc. 5·2%.

Isolation of thymine-desoxyriboside. After removal of all the adenine-desoxyriboside the water-acetone mother liquors were concentrated to a small volume. On standing for some time in the refrigerator thymine-desoxyriboside crystallized out in long needles. After one recrystallization from 50% alcohol it melted at 185°. Yield 140 mg.

Summary

A modified method, which considerably shortens the isolation of adenine-desoxyriboside from thymusnucleic acid has been described.

The product obtained differs from that already described in the literature [Klein, 1934; 1938] in having a considerably higher M.P.

Adenine-desoxyriboside isolated in this Laboratory sinters at 162–165° and melts sharply at 187–188°.

I wish to express my sincere thanks to Prof. E. J. Conway, through whose initiative this work was undertaken, for his unfailing interest and helpful criticism, and to the Irish Medical Research Council for a grant in aid of this work.

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95. THE INHIBITION OF GLUCOLYSIS BY GLYCERALDEHYDE

By LEONARD HUBERT STICKLAND

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(*Received 16 July 1941*)

THERE are three reasons why it seemed desirable to make a further study of the inhibition of glycolysis by glyceraldehyde. Firstly, from many tissues (e.g. brain, tumours, retina) whose glycolysis is completely inhibited by low concentrations of glyceraldehyde (0·001–0·005 *M*), extracts can be prepared in which glycolysis proceeds by the same series of reactions as in muscle extract. Now this series of reactions, studied in muscle extract, is only partially inhibited by even the highest concentrations of glyceraldehyde, up to 0·1 *M* [Holmes, 1934; Lehmann & Needham, 1938]. If this feebly inhibited system occurs in brain extract, why is the glycolysis of intact brain tissue readily inhibitable? Meyerhof *et al.* [1936] provided a possible explanation of this phenomenon when they showed that muscle extract contains an enzyme (aldolase) which brings about the condensation of various aldehydes with triosephosphate to form non-fermentable ketose-1-monophosphates; glyceraldehyde might in this way be rapidly removed from the system, so that no inhibition of glycolysis would be observed. Since then it seems to have been assumed that muscle extract would be inhibited by 0·001 *M* glyceraldehyde, if it were possible to maintain such a concentration in the system. However, Süllmann [1938, 1] raised strong doubts of the validity of this explanation. He confirmed that when muscle extract acts on hexosediphosphate in the presence of glyceraldehyde, the latter disappears and easily hydrolysable phosphoric esters (hexose-1-phosphates) appear; but when the substrate was glycogen no such changes could be detected. The difference presumably lay in the different concentrations of triosephosphate present in the two cases, the condensation taking place only when this concentration was high. Later in this paper two further reasons will be given for rejecting the aldolase condensation as an explanation of the absence of inhibition of glycolysis from muscle extract, and consequently a new answer to the above question must be found.

Secondly, Lehmann & Needham [1938] have shown that there is a difference between the action of freshly dissolved glyceraldehyde, which is in a dimeric form, and that of the monomeric form into which it is completely converted in the course of 3 days. The former inhibits glycolysis of glycogen in muscle extract in the same way as glucose does, the inhibition occurring at the stage

$$\text{glycogen} \rightleftharpoons \text{Cori ester},$$

while the latter has no such effect. This means that much of the work done with glyceraldehyde before 1938 must be reconsidered, as it is not known whether fresh or old solutions were used, though of course in some individual experiments it is possible to deduce this information from the results.

Thirdly, Orr & Stickland (unpublished results) have found that glycolysis in liver slices is unaffected by monomeric glyceraldehyde at concentrations up to

0·03 *M*. This glycolysis involves only the glycogen present in the liver cells, and not free glucose present in the medium [Rosenthal & Lasnitzki, 1928; Dickens & Greville, 1932; Orr & Stickland, 1941], while in those tissues in which glyceraldehyde does inhibit, the glycolysis is in every case a pure glucolysis.

These facts together suggested that it might be profitable to study the effect of glyceraldehyde on muscle extracts acting on glucose in the presence of hexokinase. This has been done before [Holmes, 1934; Needham & Lehmann, 1937, 1; Süllmann, 1938, 1, 2], but the results were contradictory; they agreed however in showing that glyceraldehyde has the same action on glycolysis in muscle extract whether the substrate is glycogen or glucose with hexokinase.

Experimental

Methods

Measurement of glycolysis. With tissue slices the usual manometric technique was used (see Berenblum *et al.* [1936] for details). The lactic acid was also determined chemically at the end of most experiments [Friedemann *et al.* 1927], and good agreement was usually obtained between chemical and manometric figures. With muscle extracts the chemical determination was relied on to give the total glycolysis, but the manometric readings were frequently used for following the time course of the lactic acid formation. The assumption that liberation of CO_2 is proportional to lactic acid formation throughout the course of an experiment ignores other minor causes of change in acidity, but cannot be a source of serious error.

Preparation of muscle extracts. Aqueous extracts of cooled rabbit muscle [Meyerhof, 1926], subjected to dialysis through collodion for 24 hr., were used in most experiments. Extracts made with 1% Na_2HPO_4 from thoroughly washed rabbit muscle [Kendal & Stickland, 1937] were also used, and the same results were obtained with the two kinds of preparation.

Hexokinase. Hexokinase was prepared from baker's yeast by the method of Meyerhof [1927]. A single preparation was used for all the experiments, as it showed no noticeable change in activity when stored for 6 months at 0° with a little toluene. 1 ml. of this solution corresponded roughly to 1 g. of pressed yeast, and 1 ml. contained 18 mg. dry weight of organic matter.

Glucose (Analar), fructose (B.D.H. 'dextrose-free') and *dl*-glyceraldehyde (Schering-Kahlbaum) were used as obtained. For use with tissue slices an isotonic solution of glyceraldehyde was prepared by dissolving it in suitably diluted Ringer's solution, and the necessary dilutions were of course made with Ringer. For the muscle extract experiments a solution in distilled water was used. The solutions were in every case allowed to stand for at least 3 days before use, to ensure that the glyceraldehyde was entirely in the monomeric form. As it is not yet settled which is the active optical isomeride, or whether in some cases both may be active, concentrations are given in this paper in terms of the *dl*-compound. Needham & Lehmann [1937, 2] and Mendel *et al.* [1938], using respectively chick embryo brei and tumour slices, agree that only the *l* form is active, but Süllmann [1938, 1], using lens extracts, found the *d*-component to be equally active.

With tissue slices at low concentrations of glyceraldehyde the inhibition of glycolysis is usually small initially, and first becomes complete after some 15 min. With muscle extracts the glycolysis only in exceptional cases follows a linear course, and sometimes ceases after some 40 min. These two facts make the

expression of the degree of inhibition difficult, and in the present work the arbitrary course has been chosen of expressing the inhibition as the average value during the first hour of the experiment.

Results

The effect of glyceraldehyde on glycolysis in liver slices. The starting point of the experiments to be described here was the observation of Orr & Stickland that glyceraldehyde does not inhibit glycolysis in liver tissue. As these results have not hitherto been published they are given in Table 1.

Table 1. *Effect of glyceraldehyde on tissue glycolysis*

In the experiments with liver slices about 40 mg. tissue were used in each vessel, with brain slices about 20 mg. and with the Daels-Biltris tumour and the oesophageal mucosa about 15 mg. The results are expressed as $Q^{N_2}_{CO_2}$ in the manometric experiments, as $Q^{N_2}_{LA}$ in the chemical. The figures for the degree of inhibition refer in the case of the liver results to the chemically determined rate of glycolysis; with the other tissues only manometric results were obtained.

	Tissue		Concentration of glyceraldehyde (*M*)					
			0	0·0003	0·001	0·003	0·01	0·03
1	Liver (rat)	Manometric	12·7	—	10·8	15·3	—	19·9
		Chemical	10·8	—	10·1	12·0	—	13·1
		Inhibition %	—	—	6	−11	—	−21
2	Liver (rat)	Manometric	13·2	—	—	—	17·3	18·2
		Chemical	10·4	—	—	—	13·7	13·3
		Inhibition %	—	—	—	—	−32	−28
3	Liver (rat)	Manometric	8·8	—	—	9·1	—	16·2
		Chemical	7·3	—	—	8·5	—	9·5
		Inhibition %	—	—	—	−16	—	−30
4	Liver (rat)	Manometric	9·1	8·9	—	—	—	17·1
		Chemical	8·3	8·2	—	—	—	12·5
		Inhibition %	—	1	—	—	—	−50
5	Liver (rat)	Manometric	4·7	4·8	4·4	8·9	10·0	11·0
		Chemical	3·6	3·3	3·3	5·4	7·2	6·0
		Inhibition %	—	8	8	−56	−100	−67
6	Liver (rat)	Manometric	4·0	—	—	7·9	14·8	—
		Chemical	4·2	—	—	7·0	9·5	—
		Inhibition %	—	—	—	−67	−126	—
7	Brain (guinea-pig)	Manometric	15·8	5·0	2·5	2·5	2·0	—
		Inhibition %	—	68	84	84	87	—
8	Brain (guinea-pig)	Manometric	14·2	—	3·3	—	—	—
		Inhibition %	—	—	77	—	—	—
9	Daels-Biltris sarcoma (guinea-pig)	Manometric	25·4	25·3	13·4	4·7	2·0	3·0
		Inhibition %	—	0	47	81	92	88
10	Daels-Biltris sarcoma (guinea-pig)	Manometric	29·0	30·4	15·4	5·6	—	—
		Inhibition %	—	−5	47	81	—	—
11	Oesophagal mucosa (rabbit)	Manometric	17·9	—	12·5	8·9	3·3	—
		Inhibition %	—	—	30	50	82	—
12	Oesophagal mucosa (rabbit)	Manometric	15·3	—	—	7·5	3·3	—
		Inhibition %	—	—	—	51	79	—

The large increase in the manometric values of $Q^{N_2}_{CO_2}$ at high glyceraldehyde concentrations has been observed by Baker [1938]. The chemical results show, however, that this increase is due only to a small extent to greater lactic acid formation, and it must be attributed to the action of aldehyde mutase, which is present in liver, and has been shown to act readily on *d*-glyceraldehyde [Dixon & Lutwak-Mann, 1937].

The results with liver are open to two interpretations. (1) The glyceraldehyde has no inhibitory action. In this case the small increase in lactic acid formation might be due either to a stimulation of the breakdown of glycogen or to formation of lactic acid from glyceraldehyde itself. The greater proportional increase when the glycogen breakdown is slower (Exps. 5 and 6, Table 1) suggests that the latter may be true. The conversion of glyceraldehyde into lactic acid is known to take place in various tissues, the reaction probably proceeding through methylglyoxal and glyoxalase [Embden & Metz, 1932; Needham & Lehmann, 1937, 2]. (2) The normal glycolysis is completely suppressed, and the whole of the lactic acid found is derived from glyceraldehyde. This cannot be the case, for in one experiment with $M/100$ glyceraldehyde the quantity of lactic acid formed exceeded that of glyceraldehyde originally present, without any allowance for the part of the latter that suffered dismutation; and in several experiments with $M/300$, a concentration which completely inhibits the glycolysis of most tissues, the lactic acid was up to four times the amount of glyceraldehyde originally present, so the glycolysis cannot have been greatly inhibited. It may be concluded that the previous interpretation is much the more likely.

This leads us to the tentative hypothesis that tissues and extracts which attack glucose show a glycolysis which is inhibitable by low concentrations of glyceraldehyde, while those which attack only glycogen (liver and muscle extract) show a glycolysis which is only partially inhibitable even by high concentrations, if at all. It is then obviously worth while to study in greater detail than has previously been attempted the action of glyceraldehyde on muscle extracts acting on glucose in the presence of hexokinase.

Some observations on the kinetics of hexokinase action. Preliminary experiments with muscle extract and hexokinase gave contradictory results (Table 2).

Table 2. *Glyceraldehyde and muscle extracts*

2·5 ml. muscle extract, 4 mg. inorganic P as a mixture of Na phosphates at *p*H 7·5, 0·26% $NaHCO_3$, $M/2000$ adenyl pyrophosphate, 0·4% starch or glucose and 0·2 ml. hexokinase solution in a total volume of 5 ml. Incubated 1 hr. at 38° in $N_2+5\%$ CO_2. Results as mg. lactic acid per 5 ml.

		Glyceraldehyde concentration (M)							
		0	0·001	0·003	0·01	0	0·003	0·01	0·03
		Glucose + hexokinase				Starch			
1		13·3	0·4	0·5	0·4	4·8	5·7	5·5	4·8
	Inhibition %	—	97	96	97	—	−18	−15	0
2		13·0	12·5	0·9	0·6	3·0	2·8	2·9	—
	Inhibition %	—	4	93	95	—	7	3	—
3		9·2	9·2	9·7	0·5	4·6	4·7	4·1	—
	Inhibition %	—	0	−5	94	—	−2	12	—
4		14·0	14·0	13·9	—	7·1	7·3	7·3	—
	Inhibition %	—	0	1	—	—	−3	−3	—
5		6·8	6·7	6·9	8·1	4·7	—	4·7	5·1
	Inhibition %	—	1	−1	−19	—	—	0	−8

There is here no inhibition of the attack on starch at a concentration of $0{\cdot}01\,M$ glyceraldehyde, and in the two cases quoted none even at $0{\cdot}03\,M$. On the other hand glucolysis is completely inhibited by $0{\cdot}01\,M$ in three experiments out of four, by $0{\cdot}003\,M$ in two out of five and by $0{\cdot}001\,M$ in one out of five. A possible explanation of these variable results was found during a brief study of the kinetics of hexokinase action, and these experiments must now be described.

If the amount of lactic acid formed in 1 hr. from glucose is determined at varying hexokinase concentrations, then a curve of the type shown in Fig. 1 (curve *G*) is obtained. The relationship is unusual in showing an abrupt appearance of full activity with a small increase in hexokinase concentration; this critical concentration of hexokinase, below which practically no glucolysis takes place, is not a fixed quantity, but varies in different muscle enzyme preparations, the range being from about 0·1 to 0·5 ml. of the standard hexokinase solution per 5 ml. with the enzymes so far studied. With fructose as substrate the critical concentration of hexokinase is lower, lying between 0·02 and 0·08 ml./5 ml. Fig. 1 shows this effect in a case where the critical concentrations were 0·08 ml. for glucose and 0·02 ml. for fructose.

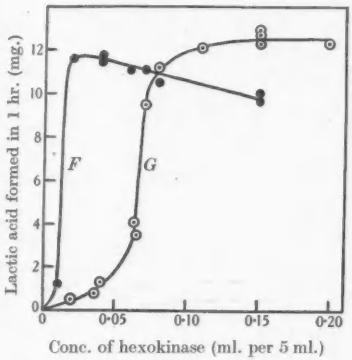

Fig. 1.

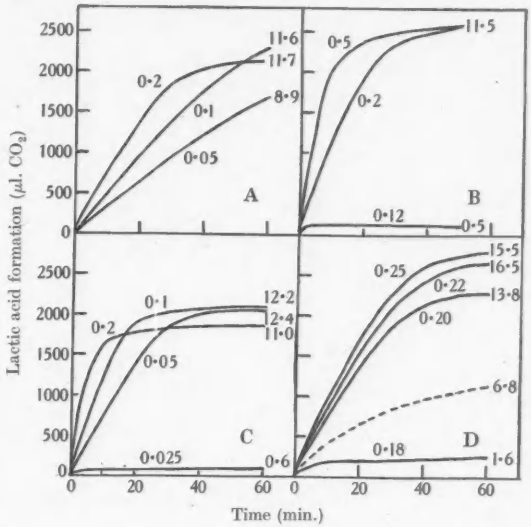

Fig. 2.

Fig. 1. The effect of increasing hexokinase concentration on glucolysis and fructolysis in muscle extract. Curve *G*, substrate glucose; curve *F*, substrate fructose.

Fig. 2. The course of glucolysis and fructolysis in muscle extract at different hexokinase concentrations. The curves were obtained manometrically, and the figure to the right of each curve gives the result of the chemical determination of lactic acid at the end of the experiment (mg./5 ml.). The hexokinase concentration (ml./5 ml.) is shown against each curve. The substrate was glucose in *A*, *B* and *D*, fructose in *C*. Curves *D* include for comparison the course of formation of lactic acid from starch (dotted line).

These results so far only deal with the total amount of lactic acid formed in 1 hr. If the time course of the reaction is followed, it is found that as the concentration of hexokinase is increased above the critical value the initial rate of glucolysis also increases, although the amount in 1 hr. remains roughly constant. Typical sets of curves showing the course of glucolysis and fructolysis at various hexokinase concentrations are given in Fig. 2. In general, it can be seen that at a concentration of hexokinase only just above the critical value the glucolysis starts relatively slowly and proceeds at a steadily diminishing rate; it may still be proceeding after 60 min. (Fig. 2 *A*) or may stop after some 40 min.

(Fig. 2, *B*, *C*). At higher concentrations, on the other hand, the glucolysis is initially much faster and very soon comes to a complete stop; the fastest case shown is in Fig. 2 *C*, where the glycolysis (in this case a fructolysis) has practically come to a standstill in 10 min. The effect of gradually increasing the concentration past the critical value is shown in Fig. 2 *D*. The cessation of the reaction at the same point at all hexokinase concentrations seems not to be due to the complete utilization of the substrate, for the amount of lactic acid formed in different experiment lies between 10 and 15 mg./5 ml., i.e. between 50 and 75 % of the glucose initially present. No explanation of these results can be offered, and they are not being further studied, as from the present point of view they are a side issue.

The importance of these phenomena in connexion with glyceraldehyde inhibition is this. In experiments with tissue slices it is commonly found that the inhibitory effect of glyceraldehyde is not immediately complete, but increases during the first 15–20 min. If the same were true in the case of muscle extract, then at high hexokinase concentrations, where the whole reaction may be finished in about 10 min., no inhibition can be expected. If any inhibition is to be found, the hexokinase concentration must be adjusted to produce a steady lactic acid formation, i.e. the concentration must be only just above the critical value.

Inhibition of glucolysis at controlled hexokinase concentration. Each muscle enzyme solution, as soon as it was prepared, was tested with a series of concentrations of hexokinase, to determine the critical value at which glucolysis first appeared. The effect of glyceraldehyde was then studied at the lowest hexo-

Table 3. *Inhibition of glucolysis in muscle extract by glyceraldehyde*

Details of experiments as in Table 1, except hexokinase concentration which is given below. Results as mg. lactic acid per 5 ml.

	Hexokinase concentration (ml./5 ml.)		Glyceraldehyde concentration (*M*)						
			0	0·001	0·003	0·01	0	0·01	0·03
			Glucose + hexokinase				Starch		
1	0·45		11·9	—	0·9	0·6	—	—	—
		Inhibition %	—	—	93	95	—	—	—
2	0·22		12·7	—	1·6	0·6	6·6	6·8	4·0
		Inhibition %	—	—	87	95	—	− 2	39
3	0·20		12·5	—	1·0	0·4	8·9	9·0	5·6
		Inhibition %	—	—	92	97	—	−1	37
4	0·50		14·1	—	0·9	0·6	—	—	—
		Inhibition %	—	—	94	96	—	—	—
5	0·20		8·7	7·6	2·1	0·7	8·0	8·7	6·8
		Inhibition %	—	13	78	94	—	−9	15
6	0·22		15·9	16·6	1·4	—	6·6	6·8	4·0
		Inhibition %	—	−5	91	—	—	−3	39
7	0·50		13·5	5·1	1·1	—	—	—	—
		Inhibition %	—	62	92	—	—	—	—
8	0·10		6·6	6·2	0·4	—	—	—	—
		Inhibition %	—	6	95	—	—	—	—
9	0·20		7·8	—	1·4	—	6·0	5·8	6·3
		Inhibition %	—	—	81	—	—	3	5
			Fructose + hexokinase				Starch		
10	0·05		13·2	—	0·7	0·5	9·3	8·5	5·8
		Inhibition %	—	—	95	96	—	8	38
11	0·015		8·0	—	1·3	0·7	6·2	6·0	4·6
		Inhibition %	—	—	84	91	—	3	27

kinase concentration which gave maximal, or in some cases slightly submaximal, lactic acid formation in 1 hr. The results of several such experiments are given in Table 3.

From these figures two facts are clear. First, 0·01 *M* glyceraldehyde has no effect on the rate of glycolysis of starch, and 0·03 *M* causes only a small inhibition (from 0 to 40 %). Secondly, 0·003 *M* glyceraldehyde causes almost complete inhibition of both glucolysis and fructolysis (on the average 90 %), while even 0·001 *M* in some cases gives a considerable degree of inhibition (Table 3, Exp. 7; cf. Table 2, Exp. 1). A curious point about these results is that a partial inhibition is rarely observed (in the whole of Tables 2 and 3 only one result lies between about 10 and 80 % inhibition). Usually when the concentration of an inhibitor is increased in threefold steps, the degree of inhibition increases slowly, as can be seen in the cases of the slices of Daels-Biltris sarcoma and of oesophageal mucosa in Table 1.

The removal of the inhibition of glucolysis by excess of hexokinase. The effect of increasing the hexokinase concentration on the degree of inhibition of glucolysis is shown in Table 4.

Table 4. *Reversal of glyceraldehyde inhibition by excess of hexokinase*

Conditions of experiment as in Table 2, except for hexokinase concentration, which is given below. Results as mg. lactic acid formed in 1 hr./5 ml.

	Substrate	Hexokinase conc. (ml./5 ml.)	Glyceraldehyde concentration (*M*) 0	0·003	0·01	Inhibition %
1	Glucose	0·10	10·7	—	1·8	84
		0·125	11·1	—	3·4	69
		0·16	11·0	—	11·5	−5
		0·20	10·8	—	10·9	−1
2	Glucose	0·22	13·1	1·3	—	90
		0·25	15·9	8·0	—	50
3	Fructose	0·02	9·6	—	1·2	87
		0·10	10·7	—	11·2	−5
		0·015	8·0	1·3	—	84
		0·02	9·6	6·3	—	34
4	Fructose	0·05	13·2	0·7	—	95
		0·20	12·3	11·9	—	3

These experiments show that, even within the range of hexokinase concentrations in which glucolysis is maximal (or nearly so), a very small change in that concentration may have a great effect on the degree of inhibition by glyceraldehyde. For instance, in Exp. 1 an increase of 60 % in the amount of hexokinase completely abolished an 84 % inhibition, and in Exp. 2 an increase of only 14 % reduced an inhibition of 90 % to one of 50 %. It has already been indicated that results of this nature are to be expected, and the explanation given of this effect is that at high hexokinase concentrations the glucolysis proceeds so fast that it is all over before the inhibiting action of the glyceraldehyde can come into play. This explanation is now seen to be insufficient. For example, in Exp. 2 (Table 4; see also Fig. 4) the small difference in hexokinase concentration caused very little difference in the course of the glucolysis; in each case the rate began to decrease after 15 min. and the reaction came to an end in 40 min., as measured by the manometric readings. The glyceraldehyde had the same opportunity to act in each, and yet the degree of inhibition was greatly different. It is clear that the hexokinase solution itself reverses the inhibiting effect of the glyceraldehyde. Whether this action is due to the hexokinase

protein or to some impurity in the solution remains undecided. Pyruvic acid at very low concentration is known to behave in this way [Mendel *et al.* 1931; see below], but determination of the total bisulphite-binding power of the preparation of hexokinase used in these experiments showed that pyruvic acid could be present only in amount quite insufficient to account for the above results.

The removal by pyruvic acid of the inhibition by glyceraldehyde of glucolysis in muscle extract. Mendel *et al.* [1931] found, using slices of Jensen rat sarcoma, that 0·001 *M* glyceraldehyde produced an inhibition of 82%. Addition of 0·00005 *M* pyruvate reduced the inhibition to 40%, of 0·0001 *M* to 20%, and of 0·001 *M* to nothing. To determine whether a similar phenomenon can be observed in muscle extract, experiments were carried out in the following way. A muscle extract was tested to find the concentration of hexokinase required to give good inhibition of glucolysis by 0·003 *M* glyceraldehyde, and then at these concentrations of hexokinase and glyceraldehyde the effect of various amounts of pyruvic acid was studied. In Table 5 the results obtained in two such experiments are given.

Table 5. *The reversal of glyceraldehyde inhibition by pyruvic acid*

Conditions of experiment, other than those given in the table, as in Table 2. Results as mg. lactic acid formed in 1 hr./5 ml.

1	Glyceraldehyde conc. (*M*)	0	0·003	0·003	0·003	0·003	0·003	—
	Pyruvate conc. (*M*)	0	0	0·003	0·0015	0·0008	0·0004	—
	Lactic acid formed	13·5	1·1	13·6	13·0	11·0	5·5	—
	Inhibition %	—	92	0	4	18	59	—
	Glyceraldehyde conc. (*M*)	0	0·001	—	—	—	0·001	—
	Pyruvate conc. (*M*)	0	0	—	—	—	0·0004	—
	Lactic acid formed	13·5	5·1	—	—	—	12·0	—
	Inhibition %	—	62	—	—	—	11	—
2	Glyceraldehyde conc. (*M*)	0	0	0·003	0·003	0·003	0·003	0·003
	Pyruvate conc. (*M*)	0	0·003	0	0·003	0·0015	0·0008	0·0004
	Lactic acid formed	15·9	16·4	1·4	16·5	17·1	15·5	15·9
	Inhibition %	—	—	91	0	0	2	0

The figures show that in Exp. 2 0·0004 *M* pyruvate (one-eighth of the inhibiting concentration of *dl*-glyceraldehyde) abolished the inhibition completely, though the same concentration had no effect on the rate of glycolysis in the absence of glyceraldehyde. In Exp. 1 the reversal was less complete at the lowest dilutions of pyruvate, but was still marked.

Now it is known that glyceraldehyde may be converted into lactic acid in some tissues [Embden & Metz, 1932; Needham & Lehmann, 1937, 2], and in particular that in muscle extract it reacts with pyruvic acid, in the same manner as phosphoglyceraldehyde, at the oxido-reduction stage in the Embden-Meyerhof scheme of glycolysis [D. M. Needham & Pillai, 1937], though only when present at a high concentration [Warburg & Christian, 1939]. Süllmann [1938, 2] found that in muscle extract the glycolysis of glycogen, inhibited by 0·1 *M* glyceraldehyde, could apparently be restored by 0·022 *M* pyruvate, but he found also that this lactic acid formation took place equally in the absence of glycogen, and must in fact be due to a direct reaction between glyceraldehyde and pyruvate. In the present case, where lower concentrations of the reactants have been employed, it appears that this explanation of the reversal of glyceraldehyde inhibition does not apply. Under the same conditions as in the above experiments (Table 5) only a trace of lactic acid was found when muscle extract was allowed to act on a mixture of 0·003 *M* glyceraldehyde and 0·003 *M* pyruvate in the absence of glucose.

The partial inhibition of starch glycolysis by high concentrations of glyceraldehyde. The view that the absence of inhibition of glycogen or starch glycolysis by low concentrations of glyceraldehyde in muscle extract is attributable to the removal of glyceraldehyde by the aldolase reaction has had great doubt cast upon it by Süllmann [1938, 1]. Two further small pieces of evidence against that view may now be mentioned.

First, it is reasonable to suppose that the faster the glycolysis is proceeding the faster will be the removal of the glyceraldehyde; i.e. if the measurements are made over some arbitrary period of time, such as 1 hr., the faster the glycolysis the less the inhibition. Exactly the contrary is found experimentally, for glucolysis, even at minimal hexokinase concentration, proceeds faster than the breakdown of starch, and yet is inhibited to a much greater extent. The assumption is made throughout, of course, that the stages in glucolysis after the primary attack on the sugar are similar to those involved in the glycolysis of glycogen.

Secondly, the condensation of triosephosphate and glyceraldehyde cannot be instantaneous, so if the above view were correct there should be an initial period during which inhibition would be observed, followed, after the removal of the glyceraldehyde, by a period of active glycolysis. Experimentally exactly the opposite is found, the inhibition increasing steadily for the first hour of the experiment. This result can obviously be observed best when the inhibition is partial (as measured over a period of 1 hr.), and results showing the course of the reaction in a few such cases are given in Fig. 3.

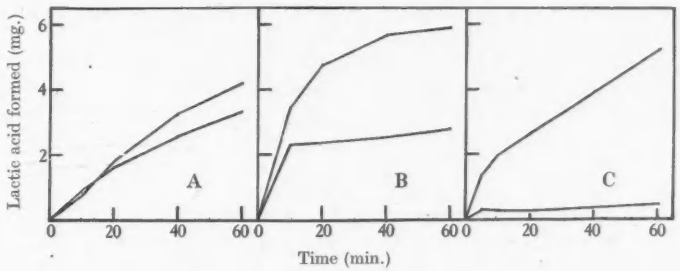

Fig. 3. The course of the inhibition of starch glycolysis by glyceraldehyde. Results by chemical determination of lactic acid. Glyceraldehyde concentrations: *A*, 0·01 *M*; *B*, 0·05 *M*; *C*, 0·03 *M*.

On the other hand, there are two observed facts which one might attempt to explain by this aldolase theory. First, the lifting of the inhibition by excess of hexokinase could be attributed to the increased rate of glucolysis, which would result in the more rapid removal of the glyceraldehyde. However, it has been shown that a very small increase in hexokinase concentration, causing only a slight increase in rate of glycolysis, is sufficient to remove the inhibition by glyceraldehyde (Table 4). Moreover, in the case in which an increase of 14 % in the hexokinase concentration reduced the inhibition from 90 to 50 % (Table 4, Exp. 2, and Fig. 4), the partially inhibited experiment showed the usual effect of the inhibition increasing with time. Secondly, when, for a variety of reasons (including age and dilution of the enzyme preparation and reduction of the phosphate concentration), the glycolysis of starch was proceeding very slowly, then this starch glycolysis was also largely inhibited by fairly low concentrations

of glyceraldehyde (0·01 *M* and sometimes 0·003 *M*). If the aldolase theory is to be rejected, then a new explanation must be found for this inhibition of starch glycolysis, and work is proceeding to try to discover its nature.

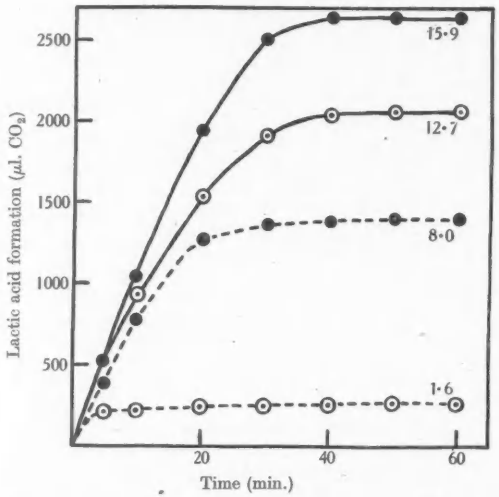

Fig. 4. The course of the inhibition of glucolysis by glyceraldehyde at different hexokinase concentrations. The curves were obtained manometrically, and the figure to the right of each curve gives the result of the chemical determination of lactic acid at the end of the experiment (mg./5 ml.). •—• indicates 0·25 ml. hexokinase solution per 5 ml., o—o 0·22 ml.per 5 ml. Dotted lines are the same inhibited by 0·003 *M* glyceraldehyde.

Discussion

The chief remaining difficulty in connexion with the inhibition of glycolysis by glyceraldehyde was that a large number of tissues, whose glycolysis is known to be inhibited by low concentrations of glyceraldehyde, have now been shown to contain the phosphorylating mechanism which is not inhibitable except by extremely high concentrations of glyceraldehyde (in red blood cells, Meyerhof [1932]; in brain, Euler *et al.* [1936], Geiger [1938]; in tumours, Boyland *et al.* [1937]; in embryo, Meyerhof & Perdigon [1939]; in retina, Süllmann & Vos [1939], Kerly & Bourne [1940]). The evidence for the presence of the whole phosphorylating system is in some cases not yet complete; for example, the disappearance of free phosphate, the need for adenosine triphosphate and coenzyme I, or the presence of the reaction between triosephosphate and pyruvic acid, or combinations of two or more of these and other such pieces of evidence, have been taken as proof of the existence of the whole system. On the other hand, there can be little doubt that further investigation will show that in every case these extracts do bring about glycolysis by the phosphorylating mechanism. Whether other mechanisms may exist as well is for the moment beside the point.

The results described here appear to go a long way towards explaining this discrepancy. They lead to the view, expressed in its simplest form, that the breakdown of glycogen to lactic acid by the enzyme system of muscle extract

(which is also present in all other tissues so far studied) is practically unaffected by glyceraldehyde, but that the breakdown of glucose by the same enzyme system is completely inhibitable. The glyceraldehyde therefore inhibits the initial stage of the process, which is catalysed by hexokinase.

The probable mode of action of hexokinase has been described by Euler & Adler [1935] and Meyerhof [1935]. The former, using a yeast preparation which they called heterophosphatese, showed that the addition of this enzyme enabled the hexosemonophosphate dehydrogenase system of Warburg & Christian to act also on hexoses. Meyerhof [1935] proved that the reaction which occurs is a stoichiometric one between hexose and adenosine triphosphate, leading to the formation of a hexosephosphate which can be converted into lactic acid by muscle extract, and that hexokinase and heterophosphatese behave in identical fashion. The effect of glyceraldehyde on this reaction remains to be studied. Iri [1939], arguing from the results of Willstätter & Rohdewald [1937] with leucocytes and yeast, thought that hexokinase must act by causing a synthesis of glycogen from glucose, and produced evidence that this synthesis does actually take place in the early stages of glucolysis in muscle extract. It seems unlikely, however, that glycogen is an intermediary in muscle glucolysis, for with optimal hexokinase concentration glucose is turned into lactic acid five times faster than is glycogen (Meyerhof [1927] and see Fig. 2), so that it would be necessary to assume the existence of a specially reactive form of glycogen as the first product of the synthesis.

Several points remain to be cleared up before this explanation of glyceraldehyde inhibition can be accepted as generally applicable. In some tissue slices, e.g. Jensen rat sarcoma [Mendel, 1929] and brain (Ashford [1934] and see Table 1), almost complete inhibition is observed with 0·001 *M* glyceraldehyde, whereas in the case of muscle glucolysis complete inhibition can be regularly obtained only with 0·003 *M*. Inhibition of muscle glucolysis by 0·001 *M* glyceraldehyde is variable and frequently absent, and the results indicate that this is possibly due to the presence in the latter cases of a slight excess of hexokinase. In some other tissue slices a higher concentration of glyceraldehyde is required to produce inhibition, e.g. chick embryo 0·005 *M* [Needham & Lehmann, 1937, 1], Daels-Biltris sarcoma 0·003 *M* and oesophageal mucosa 0·01 *M* (Table 1), and the speculation could be offered that in these tissues the effective concentration of hexokinase is higher. This reversal of glyceraldehyde inhibition by excess of hexokinase was not found by Needham & Lehmann [1937, 1] in chick embryo, but it must be remembered that they used a brei which, though free from intact cells, retained a considerable degree of cellular structure, and the hexokinase, which is a protein, may not have been able to reach the appropriate site. Their statement that 'In all cases brei made with distilled water to abolish cell integrity was used; hence it cannot be argued that hexokinase had no entrance to the glucolytic system' does not appear to me to be conclusive on this point.

Holmes [1934] and Needham & Lehmann [1937, 1] found no inhibition of glycolysis of either starch or glucose in muscle extract. They do not indicate the quantity of hexokinase used, and in the light of the present results it must be assumed that excess of this agent was present. Süllmann [1938, 2], on the contrary, found that at high concentrations of glyceraldehyde (0·05–0·07 *M*) glycolysis of glycogen or of glucose was equally inhibited (70–100 %). The explanation of this probably lies in the two facts (1) that his extracts were of very low activity (in only one experiment did the rate of glycolysis exceed 2·5 mg. lactic acid/5 ml. in 1 hr.), and (2) that the hexokinase concentration was of the

order of ten times that used in the present experiments. Glucolysis in certain other extracts has also been found not to be inhibited by glyceraldehyde, except at very high concentrations. Adler *et al.* [1937] found glucolysis by yeast preparations to be inhibited about 70% by 0·1 *M* and about 15% by 0·03 *M*, a result confirmed by Boyland & Boyland [1938].[1] The latter authors also found that in tumour extract 90% inhibition could be achieved only with 0·022 *M* glyceraldehyde, the result being the same whether the substrate was glucose, fructose or glycogen. Kerly & Bourne [1940] showed that in their extract of retina about 80% inhibition could be attained only with 0·2 *M* glyceraldehyde, but the same result was obtained even with hexosediphosphate as substrate, which indicates that at this extremely high concentration a different kind of inhibition may be involved. No attempt at a final explanation of such results can be made now, but the ready reversal of glyceraldehyde inhibition of glucolysis by excess of hexokinase may prove to be of importance in this connexion too.

Another way in which the inhibition of glucolysis in muscle extract differs from that in tissue slices is in the shape of the curve relating concentration of glyceraldehyde with degree of inhibition, this being much steeper in the case of muscle extract. Further study of the kinetics of these reactions and of the relation between hexokinase and glyceraldehyde will, it is hoped, help to show whether the present results have any bearing on the nature of glucolysis in tissue slices and its inhibition by glyceraldehyde.

Summary

Glycolysis in liver, a glycogen-fermenting tissue, is not inhibited by high concentrations of glyceraldehyde.

It is confirmed that in muscle extract glycolysis of starch is not greatly inhibited by high concentrations of glyceraldehyde, but on the other hand it is found that glucolysis by this system in the presence of hexokinase is completely inhibited by 0·003 *M* glyceraldehyde.

This inhibition is reversed by a small excess of hexokinase. It is also reversed, as is the case in slices of glucolysing tissues, by very small amounts of pyruvic acid.

[1] But in this paper it is reported that glycolysis in muscle extract is inhibited at the stage of phosphorylation of glycogen, so that at least in these particular experiments the dimeric form of glyceraldehyde was probably concerned.

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96. THE β-GLUCOSAMINASE ACTIVITY OF TESTICULAR EXTRACTS AND ITS BEARING ON THE PROBLEM OF DIFFUSING FACTORS

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Diffusing factor concentrates from testicular extracts, bacterial filtrates and snake venoms are known to decrease rapidly the viscosity of aqueous solutions of mucopolysaccharides of the hyaluronic acid type. Prolonged action of these concentrates leads to hydrolysis of the mucopolysaccharide by rupture of some of the glycosidic linkages [Chain & Duthie, 1939; 1940; McClean & Hale, 1940; 1941]. Hyaluronic acid is believed to contain equal numbers of *N*-acetylglucosamine and glucuronic acid residues [Meyer, 1938], and since it has virtually no reducing properties it probably contains equal numbers of glucosaminidic and uronidic linkages, either or both of which must be ruptured in the hydrolysis brought about by diffusing factor preparations.

It has been shown that diffusing factor concentrates from testes contain a β-glucosaminase which catalyses the hydrolysis of β-phenyl-*N*-acetylglucosaminide [East *et al.* 1941]. The same is true of snake venom and the enzyme in both cases appears to be, like the glucosaminase in emulsin [Helferich *et al.* 1933; 1934; Grassmann *et al.* 1934], specific for the β-glycosides of *N*-acetylglucosamine. Testicular extracts containing glucosaminase are unable to bring about hydrolysis of the β-glucuronides of menthol [East *et al.* 1941], borneol or pregnanediol [Meyer *et al.* 1941]. Since it is not known whether the uronidic linkages in hyaluronic acid are of the α or β type this evidence is not strong enough to rule out the possibility that an enzyme capable of hydrolysing the uronidic linkages in hyaluronic acid may also exist in testicular extracts. The methods available for the determination of *N*-acetylglucosamine residues and of the total reducing power in the partially hydrolysed hyaluronic acid are insufficiently accurate to permit of their being used to establish or disprove the existence of such an enzyme.

It has already been suggested that the effect of testicular extracts on hyaluronic acid may be due to a complex system of enzymes rather than to one enzyme only [Madinaveitia & Quibell, 1940; McClean & Hale, 1940; Meyer *et al.* 1941]. The above-mentioned specific β-glucosaminase of testis has now been shown to be distinct from the enzyme or enzymes responsible for decreasing the viscosity of hyaluronic acid solutions. There is no parallelism between the glucosaminase activity and viscosity-reducing properties of testicular extracts, and the behaviour of the glucosaminase on adsorption differs from that of the viscosity-reducing material. Crude emulsin preparations contain a specific oligo-β-glucosaminase [Zechmeister & Tóth, 1939]: it is of interest to note that a sample of emulsin containing this enzyme (B.D.H. emulsin) failed to decrease the viscosity of hyaluronic acid solutions and had no diffusing properties.

It may well be that the decrease in viscosity of hyaluronic acid solutions by testicular extracts is a process which does not involve hydrolysis of glycosidic

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linkages. In addition to such extracts, ascorbic acid [McClean & Hale, 1940], some diazo-compounds [Favilli, 1940], phenylhydrazine and H_2O_2 [Madinaveitia & Quibell, 1941, 1] all have a similar effect. It is difficult to ascribe hydrolytic properties to some of these reagents. Moreover, treatment with alkali irreversibly reduces the viscosity of hyaluronic acid [East *et al.* 1941] although glycosidic linkages are as a rule unaffected by this reagent.

Experimental

Estimation and properties of β-glucosaminase

The substrate used, β-phenyl-*N*-acetylglucosaminide, was incubated with the enzyme preparation to be tested and the degree of hydrolysis determined by means of the Folin & Ciocalteu [1927] phenol reagent. This technique has been successfully applied in the study of glucosaminases from other sources [Neuberger & Pitt Rivers, 1939]. The alternative procedure, estimation of liberated *N*-acetylglucosamine by the method of Morgan & Elson [1934] was found to be somewhat unreliable.

Quantitative estimation. A solution of β-phenyl-*N*-acetylglucosaminide (1 ml., containing 10 mg.) in *M*/6 acetate buffer *p*H 4·6 mixed with an aqueous solution (1 ml.) of the enzyme preparation is incubated at 37° for 18–24 hr. To the incubated mixture Folin & Ciocalteu's phenol reagent (5 ml.) is added and the precipitated proteins removed. To a known volume (5 ml.) of the clear solution saturated Na_2CO_3 (5 ml.) is added, the colour allowed to develop for 30 min. or more and the liberated phenol determined colorimetrically by comparison with standard solutions of phenol treated in the same way. The blanks obtained using dialysed and moderately active enzyme preparations (containing not more than 10 mg. solids per ml.) are negligible.

Good recovery is obtained when known amounts of phenol are mixed with a dialysed testicular extract (10 mg. solid per ml.):

Phenol present (mg./ml.)	0·346	0·277	0·241	0·138
Phenol found (mg./ml.)	0·345	0·266	0·238	0·134

Up to 10% the degree of hydrolysis of the substrate (24 hr. at 37°) is practically proportional to the concentration of enzyme.

Dialysed testis extract

mg. enzyme/ml.	0·4	0·8	1·2	1·6	2·0
% hydrolysis	3·9	5·7	8·7	10·0	11·2
Ratio	0·10	0·14	0·14	0·16	0·18

Crude Crotalus atrox *venom*

mg. enzyme/ml.	3	5	10	20
% hydrolysis	0·6	1·0	1·6	3·7
Ratio	5·0	5·0	6·2	5·3

The degree of hydrolysis is virtually a linear function of time, and therefore the length of incubation is not critical, provided that it is the same for all samples compared in each particular experiment.

Time (hr.)	1	2	3·5	5	7	20
% hydrolysis	0·8	1·0	1·6	2·3	2·5	8·8
Ratio	1·25	2·0	2·5	2·2	2·8	2·3

p*H optimum*. The *p*H chosen for the quantitative estimation is the optimum for glucosaminase activity. To determine it constant amounts of a dialysed testicular extract (2 mg.) and of substrate (10 mg.) were incubated for 18 hr. at 37° in the presence of acetate buffers (2 ml.) of varying *p*H.

*p*H	3·6	3·8	4·0	4·2	4·4	4·6	4·8	5·8
% hydrolysis	2·7	2·7	4·0	4·0	4·9	6·8	6·7	5·5

Effect of NaCl. This salt has a great effect on the reaction by which the viscosity of vitreous humour is decreased [Madinaveitia & McClean, 1940; McClean & Hale, 1940; Madinaveitia & Quibell, 1941, 2], but very little on the action of glucosaminase. Constant amounts (2 mg.) of a dialysed testicular extract were dissolved in NaCl solutions of various concentrations (1 ml.) and mixed with the buffered standard substrate solution. After 18 hr. incubation at 37° the degrees of hydrolysis attained were:

Molarity NaCl	0·000	0·001	0·005	0·01	0·05	0·1	0·5	1·0
% hydrolysis	10·6	12·6	13·0	13·0	11·1	10·4	10·7	8·0

Effect of substrate concentration. Solutions of β-phenyl-*N*-acetylglucosaminide (2 ml.) of various concentrations in *M*/6 acetate buffer were incubated for 18 hr. at 37° with a solution of dialysed testicular extract (0·5 ml., 6 mg./ml.).

Concentration of substrate (mg./ml.)	4	3	2	0·8	0·6	0·4	0·2
% hydrolysis	13	17·5	24	30	35	40	50

Comparison of the viscosity-reducing and glucosaminase activities of various testicular diffusing factor preparations

The viscosity-reducing powers [Madinaveitia & Quibell, 1940] and the glucosaminase activities of various diffusing factor preparations were compared with those of the standard diffusing factor preparation:

	Viscosity reducing power	Glucosaminase activity
Standard [Madinaveitia, 1938]	1	1
Standard [Bacharach *et al.* 1940]	4·0	0·1
Preparation DF 8B (Glaxo Laboratories, Ltd.) dialysed testis extract	2·7	0·7
$(NH_4)_2SO_4$ purified [Madinaveitia, 1939]	4·4	0·6
$(NH_4)_2SO_4$ and Pb acetate purified	20·3	0·74

It is clear that no parallelism exists between the two kinds of activity and that $(NH_4)_2SO_4$ treatment followed by Pb acetate fractionation [Madinaveitia, 1941] yields a mucinase preparation practically free from glucosaminase.

Kaolin adsorption. The mucinase from dialysed testicular extracts is more readily adsorbed than the glucosaminase from this source. A solution of dialysed testicular extract (5 ml., 7 mg./ml.) was adsorbed using various amounts of kaolin (B.D.H. acid-washed) for 30 min. at room temperature. After removing the adsorbate the N content, the glucosaminase activity and the viscosity-reducing power of the clear supernatants were estimated.

	Kaolin, g.				
	0·05	0·1	0·2	0·4	0·8
	% adsorbed				
Mucinase	0	5	15	69	100
Glucosaminase	0	0	0	0	60
Nitrogen	6·6	—	27·6	50·5	93·4

With more dilute enzyme solutions this effect is more marked. Solutions of the same preparation (4 ml., 2 mg./ml.) were treated with kaolin as above.

	Kaolin, g.				
	0·019	0·037	0·075	0·15	0·3
	% adsorbed				
Mucinase	28·5	47·5	65·7	100	100
Glucosaminase	0	0	0	90·7	96

Comparison of kaolin with other adsorbents. Solutions of the above enzyme preparation in *M*/5 acetate buffer *p*H 5·0 (4 ml., 2 mg./ml.) were treated with the adsorbent under examination (0·75 g.). After centrifuging, the mucinase and the glucosaminase contents of the supernatants were determined.

	% adsorbed		
Adsorbent	Mucinase	Glucosaminase	Ratio
Kaolin	60	14	4·3
Fuller's earth	68	24	2·8
Bauxite	45	18	2·5
Kieselguhr	51	8	6·4
Frankonite	84	38	2·2

Action of HCl and of NaOH on the viscosity of vitreous humour preparations

Two samples (40 ml. each) of a solution in *M* NaCl of a vitreous humour preparation (0·25 %) were mixed with the same volume (10 ml.) *M* HCl and *M* NaOH respectively and kept in a thermostat at 49·2°. Samples (5 ml.) of each solution were withdrawn at intervals. To those containing HCl, Na acetate (2 ml., *M*) was added and those containing NaOH were buffered with acetic acid (2 ml., *M*). The specific viscosity of the buffered solutions was then determined at 25°. The comparison was thus carried out at about the same *p*H and in the presence of comparable concentrations of NaCl.

	Sample kept in	
Time of reaction min.	HCl	NaOH
	Specific viscosity	
0	0·295	0·249
5	0·254	0·203
10	0·240	0·183
15	0·222	0·170
20	0·210	0·162
30	0·207	0·145
40	0·177	0·137
80	0·147	0·095
160	0·109	0·070
1140	0·055	0·012

SUMMARY

Diffusing factor concentrates from testicular extracts contain a specific β-glucosaminase. This enzyme is different from the agent present in these concentrates which rapidly decreases the viscosity of mucopolysaccharides of the hyaluronic acid type.

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97. THE ABSORPTION AND EXCRETION OF 'MINOR' ELEMENTS BY MAN

2. COBALT, NICKEL, TIN AND MANGANESE

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In a previous paper Kent & McCance [1941] described the results of their work on the absorption and excretion of Ag, Au, Li, B, and V. The experiments now to be described have been carried out in a similar way. As before, the subjects have been either patients or normal persons and the same spectrochemical apparatus and analytical technique have been used. The previous article should be consulted for information about the analytical methods; a full description of the normal subjects and of the metabolic organization has been given by McCance & Widdowson [1941].

Cobalt

Bertrand [1926] and his co-workers showed that Ni and Co were present in all samples of arable soil collected from European countries. They also found these elements in plants and in many human and mammalian organs [Bertrand & Macheboeuf, 1926; Bertrand & Mokragnatz, 1925]. Wohlwill [1907] reported that neither Ni nor Co was absorbed from the gut, but this must have been due to faulty analysis, for absorption has been demonstrated by others [Mascherpa, 1927; Simesen, 1939]. The facility with which 'coast' disease in sheep can be cured by a Co drench [Askew & Dixon, 1936; Wunsch, 1937] and the ease with which polycythaemia can be produced by the oral administration of Co [Josland, 1936] are excellent proofs that at any rate small quantities must be absorbed. If Co has once been absorbed or has been given parenterally, the literature suggests that it is excreted partly by the kidney and partly by the intestine and that the route depends to a large extent upon the nature of the compound and upon how it has been administered. Untersteiner [1931], for example, found that divalent Co was more rapidly eliminated than trivalent Co. Simesen [1939] recovered in the urine of the next 24 hr. 80% of the Co which he had injected as $[Co_3CO(NH_3)_4]Cl$ subcutaneously into rabbits. This compound was excreted unchanged by the kidney. Mascherpa & Perito [1931], who administered $CoCl_2$ to guinea-pigs by the same route, recovered from the urine during the following 10 days less than half the quantity injected. Le Goff [1927] injected 24 mg. of $CoCl_2$ intramuscularly into a man and recovered 6·8 mg. of the salt in the urine within the next 18 hr. He recovered a much smaller quantity (2·64 mg.) from another patient who was a diabetic. Unfortunately the faeces were not examined.

The presence of Co in bile was demonstrated long ago by Stuart [1884]. This was not confirmed by Mascherpa [1927], but has been substantiated by Caujolle [1936] after intravenous administration of the chloride.

The subject of the present experiment was a male hospital patient suffering from carcinoma of the stomach. His kidneys were functioning normally and he

did not vomit during the studies. Excluding the preliminary and after periods the experiment lasted 2 weeks. During the first, which served as the control, the patient was given a weighed diet, and urine and faeces were collected quantitatively. During the 2nd week the diet was repeated in every possible respect and urine and faeces were collected as before. On 5 of the 7 days of the 2nd week $CoCl_2$ was injected intravenously and in all 13 mg. of Co were administered in this way. The urine and faeces of both weeks were analysed for Co, but not the food. The results are given in Table 1. The 1st week's data show that the food

Table 1. *The metabolism of cobalt*

Week	Co injected intravenously mg.	Co excreted, mg.			Urine Co as % of total
		Urine	Faeces	Total	
1	0	0·21	1·04	1·25	17
2	13	2·36	1·78	4·14	57
Excess of 2 over 1	13	2·15	0·74	2·89	74

must have contained appreciable amounts of Co and that only 17 % of it passed through the kidney into the urine. During the week in which the injections were given there was a tenfold increase in the urinary excretion and a much smaller increase in the faecal excretion. Assuming the Co intake by mouth to have been the same in both weeks, the results show that of the 13 mg. which were injected intravenously 2·89 were excreted in the week, 74 % of this amount by the kidney. This single experiment confirms in broad outline the results of previous workers on animals and of Le Goff [1927] on man. It suggests that the gut is the main channel of excretion for the Co in natural foods, probably because relatively little is absorbed. Once Co has reached the tissues, however, it indicates that the processes of elimination are very slow, and that the kidney is the organ chiefly responsible. In the rate at which the Co was excreted the present results differ from those of Copp & Greenberg [1941], who administered radioactive Co to two rats and found that 90 % of the Co injected intraperitoneally into one animal was excreted within 4 days. This rapid elimination may be peculiar to the rat.

Nickel

The occurrence and distribution of Ni in soil, plants and in human and animal organs was studied by Bertrand and his fellow workers [Bertrand & Macheboeuf, 1925; 1926; Bertrand, 1926]. They found that Ni and Co had similar distributions in nature, but that animals tended to contain more Co than Ni, plants and soils more Ni than Co [Bertrand & Macheboeuf, 1926]. Mascherpa [1927] and others have studied the absorption and excretion of Ni and its salts. Large doses, whether by mouth or injection, produce a muco-haemorrhagic enteritis, and there is general agreement that this metal is excreted into the intestine rather than by the kidney. This is thought to explain why early workers found that Ni was not absorbed after being taken by mouth. It has been demonstrated in the bile by Stuart [1884], Lehmann [1909] and Caujolle [1937]. Few records have been found of experiments on man, and none of the elimination of Ni after intravenous or subcutaneous administration. A general account of the absorption, excretion and pharmacology of both Ni and Co has been given by Hendrych & Weden [1934].

Two normal men were the subjects for the present studies. Each received daily intravenous injections of $NiCl_2$ during the second week of a long metabolism experiment. The results are shown in Table 2, and it will be seen that before the

Table 2. *The absorption and excretion of nickel*

Subject	Length of period days	Ni intake mg./period: Injected	Ni intake mg./period: In food	Ni excretion mg./period: In urine	Ni excretion mg./period: In faeces	Balance mg.	Ni 'recovered' mg.
E. B.	7		2·25	1·67	0·72	− 0·14	
	7	9	1·99	3·42	0·69	+ 6·88	3·8
	7		2·67	3·16	1·19	− 1·68	
N. K.	7		—	2·29	1·20	—	
	7	20	5·51	6·70	1·33	+17·48	
	4		2·21	2·88	0·59	− 1·26	
	7		3·43	3·48	2·11	− 2·16	7·4
	7		3·20	2·61	2·05	− 1·46	

injections were made there was more Ni in the urine than in the faeces. This suggests a reasonably good absorption of the traces of Ni normally present in food. E. B. was almost exactly in balance at this time. N. K.'s balance cannot be given since the figure obtained for his food suggests that it became contaminated with Ni from cutlery during its preparation for analysis. Great precautions were subsequently taken to work only with wooden utensils. During the period of injection the output of Ni rose in the urine of both subjects, so that they eliminated more by this channel than they took in with their food, and they continued to do so in later periods. Clearly Ni was being excreted in the urine. The faecal outputs did not rise during the period of Ni injections, and in subsequent periods they varied in quantity but tended to be slightly higher than they had been during the preliminary week. Only 42 % of the Ni injected into E. B. was recovered and 37 % of that given to N. K. Ni, like Co and Sn (*vide infra*), was excreted slowly and rather incompletely in these experiments and the organ mainly concerned was the kidney, not the gut.

Tin

The metabolism and pharmacology of Sn have been studied spasmodically since foods began to be preserved in cans. Most authors are in agreement that foods may become contaminated with Sn from tinned containers, but small quantities of the metal have also been found in fresh foodstuffs [Boyd & De, 1933; Bertrand & Ciurea, 1931]. Within recent years Sn salts have had a vogue in the treatment of furunculosis and most of the proprietary preparations are intended to be given subcutaneously. Apart, therefore, from the possibilities of industrial poisoning, it is evident that the body is frequently faced with the necessity of having to deal with small amounts of Sn salts. Buchanan & Schryver [1908], using human subjects, and Datta [1940], working with rats, came to the conclusion that tin was poorly absorbed and that small amounts taken by mouth were excreted mainly in the faeces. Sn given by subcutaneous or intravenous injection to animals has been reported to be rapidly removed from the circulation and to be excreted slowly and incompletely by the kidney [Ungar & Bodlander, 1887; Buchanan & Schryver, 1908; Salant *et al.* 1914; 1918; Salant, 1920]. No account has been found of the excretion of Sn after intravenous or subcutaneous administration to man.

Two normal men acted as subjects for the present investigations and each received a total of 28 mg. of Sn as 'Stanoxyl' by daily intravenous injection during the 2nd week of a 21-day metabolism experiment. The 1st week served as a control, during which the absorption and excretion of the Sn present in the food was followed. The results are given in Table 3. Both men were roughly in

Table 3. *The absorption and excretion of tin*

Subject	Length of period days	Sn intake mg./period: Injected during 2nd week	Sn intake mg./period: In food	Sn output mg./period: In urine	Sn output mg./period: In faeces	Balance mg./period	Sn recovered mg.
E. B.	7	0	14·4	7·2	6·6	+ 0·6	
	7	28	13·1	12·6	7·3	+21·2	19·7
	7	0	12·4	13·4	11·9	−12·9	
R. M.	7	0	12·8	8·8	2·9	+ 1·1	16·7
	14	28	30·5	36·6	10·6	+11·3	

balance during period 1 and were excreting 52 and 75 % of their whole outputs of Sn in the urine. These figures are much higher than those of Datta [1940] or of Buchanan & Schryver [1908]. During subsequent periods the injected Sn was slowly excreted. 70 % of the amount administered was recovered from E. B. and 60 % from R. M. The kidney excreted most of this extra Sn, although the amount in the faeces also rose slightly. The part played by the kidney can be appreciated more fully if the amounts in the food and urine are compared before and after the injections were given.

Manganese

The regular occurrence of Mn in plants and animals—and hence in food—has been a well-established fact for many years [Bertrand, 1939], and many tables have been published showing the usual range of Mn concentration in various foodstuffs [Lindow & Peterson, 1927; Skinner & Peterson, 1928; Davidson, 1929; Remington & Shiver, 1930; Richards, 1930; Peterson & Skinner, 1931]. Perla *et al.* [1939] found that rats might retain little or none of the Mn naturally present in the food, practically all of it passing out in the faeces. When inorganic Mn was added to the food so that the intake was raised from 0·096 to 0·165 and later to 13·45 mg./rat/day, 25 % of the dose was absorbed and retained. Skinner *et al.* [1931] obtained somewhat similar results, but found much higher percentages of Mn normally excreted in the urine.

The kidney has never been found to play an active part in the elimination of Mn [Harnack, 1901]. Normally, only a very small part of the Mn in the food is excreted by this organ, and the figure of 20 %, which Skinner *et al.* [1931] obtained, seems very high in the light of other work. Mn taken by mouth may increase slightly the amount in the urine, but the proportion so excreted always falls [Perla *et al.* 1939; Skinner *et al.* 1931]. Reiman & Minot [1920] showed that these conclusions held good also for human subjects, but in their opinion part at least of the Mn in the faeces represents Mn that has been absorbed and subsequently eliminated. These workers found that after a man had taken 8 g. of franklinite, containing 0·77 g. of Mn, the blood Mn might rise from 0·012 to 0·024 mg./100 ml. within an hour, and that after patients with biliary fistulae had taken 5 g. of franklinite by mouth the Mn in the bile might rise to 10 times its previous level. They held therefore that at least a part of the Mn in the faeces represented metal which had been absorbed, only to be re-excreted. Few references have been found to experiments in which Mn was injected into animals and none to such experiments on man. Cahn [1884] injected toxic doses of Mn salts into rabbits, killed the animals shortly afterwards and analysed their organs. He concluded that Mn so administered was eliminated mainly by the intestine. Quite recently Greenberg & Campbell [1940] have used a radioactive

isotope, Mn^{54}, to follow the fate of Mn in the body. 1 mg. of Mn^{54} was injected intraperitoneally into 1 rat and during the subsequent 4 days 90·7 % of it was recovered in the faeces. The result would have been more convincing if the Mn had been given intravenously or subcutaneously rather than intraperitoneally. The quantity excreted in the urine was too small to have any significance and the remaining 9·3 % of the injected Mn^{54} was found in the bodily organs.

One woman and two men—all normal persons—were the subjects of the present experiments. Each received 4–7 injections of Mn butyrate during the 2nd of a 4-period metabolism experiment. E. B. received a diet designed to contain very little Mn; A. M. and P. S. took 40–50 % of their calories in the form of white flour. In other respects the latter's diets were freely chosen. A. M. (the woman), who received 14·3 mg. of Mn, and E. B., who received 19·8 mg., excreted little or none of the injected dose within the time of observation. P. S., however, who received 31·5 mg., probably excreted 16·1 mg. in the faeces during the last 3 weeks of his experiment, but the balances were somewhat irregular and unconvincing. The full results are to be found in Table 4.

Table 4. *The absorption and excretion of manganese*

Subject	Period no. and length	Mn intake mg./period		Mn excretion mg./period		Balance mg./period	Mn recovered mg.
		By mouth	Injected	Urine	Faeces		
A. M.	1 (7 days)	15·5		0·7	13·7	+ 1·1	
	2 (7 days)	19·7	14·4	0·4	17·5	+16·1	
	3 (7 days)	23·4		0·3	24·9	− 1·8	3·1
	4 (7 days)	15·8		0·9	18·0	− 3·1	
E. B.	1 (6 days)	10·3		0·3	10·3	− 0·3	
	2 (6 days)	13·4	19·8	0·2	14·5	+18·5	
	3 (6 days)	12·5		0·4	12·0	+ 0·1	0
	4 (6 days)	14·6		0·4	8·2	+ 6·0	
P. S.	1 (7 days)	46·5		1·2	48·0	− 2·7	
	2 (7 days)	43·5	31·5	0·3	59·0	+15·7	
	3 (7 days)	32·4		0·1	37·6	− 5·4	16·1
	4 (7 days)	30·5		0·3	25·1	+ 5·1	

In no subject was there any evidence that any of the injected Mn was excreted in the urine. So far as the urine is concerned these results are essentially in agreement with those of most previous workers. It is evident, however, that Mn is not excreted freely, if at all, by the human bowel when it is injected in the amounts used for these experiments. The partial excretion shown by P. S. may have been the result of the larger doses he received, or of the smaller storage capacity which he possibly possessed. At any rate the difference is thought to have been genuinely one of metabolic behaviour and not to have arisen from contaminations or analytical inconsistencies. The Mn in the foods and faeces of P. S. and in some of the specimens of A. M. was determined chemically by the periodate method of Willard & Greathouse [1917]. The results agreed satisfactorily with those obtained spectrographically, and actually the figures given for P. S. in Table 4 were those obtained by the chemical method.

Table 4 shows that the Mn intakes may vary considerably on natural foods. One of the easiest ways of raising them is to eat a large amount of brown bread, for bran is very rich in Mn. 100 g. of 92 % flour were found to contain 2·15 mg. of Mn and 100 g. of 69 % flour only 0·49 mg. Table 5 shows the balances of 2 persons when they were deriving 40–50 % of their calories from 69 % flour and from 92 % flour.

Table 5. *Manganese intakes and excretions on diets containing large amounts of white and brown flour*

Subject	Diet	Mn intake in food mg./week	Mn excretion, mg./week			Balance mg./week
			Urine	Faeces	Total	
A. M.	White flour	15·5	0·7	13·7	14·4	+1·1
	Brown flour	59·6	0·2	56·2	56·4	+3·2
E. W.	White flour	18·8	0·4	19·2	19·6	−0·8
	Brown flour	61·8	0·6	60·8	61·4	+0·4

Taking the results in Tables 4 and 5 together, they show that the urinary excretion of Mn, like that of Fe, is negligibly small, whatever the intake by mouth or injection. The fact that injecting Mn in these doses did not necessarily provoke any excretion of the metal by either kidney or gut recalls that the human animal has been shown to react to injections of Fe in exactly the same way [McCance & Widdowson, 1938], and illustrates the biochemical and pharmacological affinities of the two metals, some of which were pointed out long ago by Cahn [1884].

Summary

Metabolism experiments on men and women, combined with intravenous injections of Co, Ni, Sn and Mn salts, have shown that

(1) One man excreted about 20 % of his food Co in the urine. Injected Co was excreted slowly, mainly by the kidney.

(2) Two men excreted 60–70 % of their food Ni in the urine. Injected Ni was excreted slowly, mainly by the kidney.

(3) Two men excreted between 50 and 80 % of their food Sn in the urine. Injected Sn was excreted very largely by the kidney.

(4) Only a very small part of the Mn in the food was excreted in the urine, and there was no increase after the intravenous injections of Mn salts. Two persons retained the whole of the injected Mn, a third excreted about 50 % by the bowel.

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98. THE BIOCHEMISTRY OF BACTERIAL TOXINS

1. THE LECITHINASE ACTIVITY OF *CL. WELCHII* TOXINS

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Until recently the specific toxic substances produced by bacteria have been investigated mainly by pathological and serological methods and further knowledge of their chemical nature and modes of action is clearly desirable. In addition to the intrinsic value of such knowledge there would be an immediate practical advantage to be derived from the purification and isolation of the specific toxins from toxic culture filtrates for use in the preparation of antitoxic sera, particularly if the toxins are mixtures of several antigens, as for instance in the *Cl. welchii* group. In these cases a full complement of antibodies to all the toxic components of the invading organisms is the goal in serum therapy, but the response to immunization even with a single antigen is variable in individual animals and it is not necessarily the case that with a mixture of antigens the relative proportions of the different antibodies in the serum will reflect those of the corresponding antigens in the toxin or toxoid used for immunization.

The isolation of particular toxic components would also be an advantage in studying the mode of action of the toxins on the cell. That a toxin may have an affinity for a particular type of tissue or cell is well known, and in some cases the physiological effect of a particular toxic component in a complex toxin has been demonstrated by the use of suitable antitoxic sera to inhibit the effect of the other components, but little is known of what these effects mean in terms of cell chemistry. In searching for the biochemical mechanisms by which the bacterial toxins affect the cells of the host two main hypotheses may be considered.

First, the toxin may be a substance which blocks a metabolic reaction in the host by competing with the normal substrate for the enzyme catalysing this reaction; this hypothesis is the counterpart of the theory of Fildes [1940] concerning the mode of action of chemotherapeutic substances, according to which the chemotherapeutic agent blocks an essential metabolic reaction in the invading organisms. This conception has already received some attention. Wooldridge & Higginbottom [1938] showed that the α-toxin present in *Cl. welchii* toxins inhibited the aerobic oxidation of succinate by various minced tissues of the guinea-pig, and that this inhibition was in turn considerably decreased by the specific antitoxin; they did not, however, detect which of the various enzyme systems concerned in the oxidation was inhibited. Peters & Cunningham [1941] have examined the action of diphtheria toxin on tissue respiration, and on various dehydrogenase and phosphorylation systems, but none of the enzymes examined was affected by the toxin.

The second hypothesis is that the toxin itself may be an enzyme exerting its toxic function by attacking, according to its degree of enzyme specificity, one or more substances which are normal constituents of a cell. It consequently distorts the metabolism of the cell in one or other particular direction, either primarily by destruction of an essential structure or the inhibition of a metabolic mechanism or secondarily by the production from a normal cell constituent of a toxic substance with such powers. On this hypothesis the toxin can primarily affect only those cells which contain the appropriate substrate for its enzymic activity and then only if this substrate is accessible to it. It is of course well known that certain bacterial culture filtrates have enzymic activity, but such activity has not in general been related to their toxic effects. The hydrolysis of lecithin by snake venom with the formation of lysolecithin has been for long the only instance in which the action of a toxin on a pure substance *in vitro* affords a satisfactory basis for its effect *in vivo*, namely haemolysis; for it is known that the red blood cells contain lecithin and that lysolecithin is a highly haemolytic substance. Recently the presence in various bacterial culture filtrates and in snake and spider venoms of an enzyme, hyaluronidase [Chain & Duthie, 1940], which rapidly reduces the viscosity of certain mucoproteins, has been correlated with that of a 'diffusing factor' which has a marked effect on the permeability of skin. McClean & Hale [1941] have pointed out the possible relationship of this enzyme to the local invasiveness of the organism. There is thus already evidence that certain toxins contain enzymes which may contribute to the pathogenic effect.

The basis of both these hypotheses of the modes of action of bacterial toxins is that the initial lesion in the host cell is one of molecular dimensions and high chemical specificity. Since a distortion in the metabolism of one type of cell or group of cells may affect the metabolism of neighbouring cells or tissues a characteristic pathological picture may develop throughout the tissues of the host as a result of this primary lesion. The total pathogenic effect of a bacterial infection is no doubt a summation of all the metabolic disturbances caused by the growth of the micro-organism, including those due to serologically non-specific toxic substances, for example histamine.

Recent advances in our knowledge of the biochemistry of micro-organisms permit a rational approach to these problems on several interrelated lines. The separation of the individual toxic components should be easier if the culture medium contains the minimum amount of substances similar to these toxic factors in chemical nature, and even more so if the proportion of a particular toxic component can be increased or decreased at will. The results of recent work on bacterial metabolism point to the possibility that the amount and nature of the toxic substances excreted by the bacteria are, like their content of endogenous enzymes, largely determined by the conditions in which the bacterial growth is taking place. Modern work on bacterial nutrition has demonstrated the effect of the composition of the culture medium on bacterial growth and made possible the use of relatively simple culture media of known chemical composition. It is therefore now easier to ensure that the toxin production shall take place under controlled conditions which can be varied at will. The value of this approach to the problem has been strikingly exploited by Mueller, Pappenheimer and co-workers [see Mueller, 1940; Mueller & Miller, 1941; Pappenheimer, 1937] in the case of diphtheria toxin. Its application to antigenically complex toxins would however be greatly facilitated by a knowledge of the biochemical action of the separate toxic components, if such knowledge made possible the substitution of a rapid specific chemical reaction for the complicated

animal tests necessary in the serological method. Moreover, the biochemical action of the toxin might give some clue to a variation in the culture medium likely to enhance the yield of a toxin, as in the analogous case of adaptive enzymes.

Because of the importance of *Cl. welchii* in war-wound infections it was decided to begin this investigation by examining the enzymic activity of the toxic culture filtrates of this organism. The present paper gives an account of a method used for the routine production of *Cl. welchii* (Type A) toxin[1] and of a lecithinase in the toxin which is probably identical with the lethal factor, α-toxin. The detection of this lecithinase was a direct consequence of the work of Dr R. G. Macfarlane and his colleagues on the action of the α-toxin of *Cl. welchii* on egg-yolk emulsions, following on the observations by Nagler [1939] that this toxin had a specific action on human serum.

Cl. welchii *toxins*

Wilsdon [1931; 1932–33] examined the causative organisms of several diseases and showed that they could be classified into 4 types of *Cl. welchii*, A, B, C and D, differing from each other serologically. The toxins produced by these four types are different though they have one or more toxic factors in common; the nomenclature and distinguishing effects of these components are set out in Table 1.[2] The differentiation of these toxic factors has been made

Table 1. *Antigenic analysis of* Cl. welchii *toxins*

Type	Disease	Toxins α	β	δ	ε	θ
A	Gas gangrene	+ + +	−	−	−	+
B	Lamb dysentery	+	+ + +	±	+ +	−
C	'Struck' in sheep	+	+ + +	+	−	−
D	'Pulpy kidney' in sheep	+ +	−	−	+ +	−
Characteristics of toxins:		Lethal Haemolytic Dermo-necrotic	Lethal Dermo-necrotic	Haemolytic ? Lethal	Lethal Necrotic	Haemolytic Lethal ? Dermo-necrotic

almost entirely by serological methods, chiefly by the work of Glenny and his colleagues [Glenny *et al.* 1933], although Prigge [1937] obtained from Type A filtrates, by fractional salting-out with Na_2SO_4 and $(NH_4)_2SO_4$, fractions with different proportions of the α- and θ-toxins, and thus demonstrated that at least two different substances were involved. Recently van Heyningen has achieved a considerable purification of the α-toxin from Type A filtrates and obtained it free from θ-toxin; this will be described in a paper to be published in a later issue of this Journal.

Nagler [1939] observed that when *Cl. welchii* was grown in human serum an opalescence developed and eventually a layer of fat rose to the surface of the liquid medium. This phenomenon took place with the exogenous toxin from all

[1] The word toxin is used in this paper to cover the unfractionated toxic culture filtrates, which may contain also immunologically non-specific substances. The various antigenically distinct factors will be specifically designated; thus α-toxin, and the corresponding antibody as α-antitoxin or anti-α serum.

[2] It was agreed at a representative colloquium held at Cambridge in April 1941, under the chairmanship of Prof. T. Dalling, that Glenny's original description of the lethal, haemolytic and dermonecrotic factor in Type A filtrates as α-toxin should be adhered to by English workers; and that the second haemolytic toxin in these filtrates should be called θ-toxin [cf. Smith, 1941; Stephenson & Dalling, 1941].

types of *Cl. welchii* but not with other bacterial filtrates, and was specifically inhibited by *Cl. welchii* antitoxin. He found that the activity in this respect ran parallel with the lethal action of type A toxins and that it could be used in an end-point method for the titration of the appropriate antitoxin. Later he showed [Nagler, 1941] that the reaction could be used to estimate the combining power (toxin + toxoid) of a toxin, or of toxoid alone, with antitoxin. Macfarlane *et al.* [1941] found that *Cl. welchii* toxin acts on a clear emulsion of egg-yolk in saline to give a similar but more marked opalescence and finally a cream due to the coalescence of fat globules. They showed that this reaction was due entirely to the α-toxin, which is the lethal factor predominant in type A filtrates and that β-, δ-, ε- and θ-toxins had no effect. They found also that this egg-yolk reaction and the haemolysis caused by α-toxin are activated by Ca^{++} ions and remarked that 'the strict parallelism between haemolysis by α-toxin and the production of free lipoid from lecitho-vitellin led us to suppose that both these activities might be manifestations of the same enzyme reaction'.

These authors considered that the biochemical action of the toxin was to liberate fat from combination in a lipoprotein. We have found however that after the action of the toxin the whole of the original lipin P is present as a water-soluble compound, while the protein and neutral fat are almost unchanged chemically. The toxin also decomposes an aqueous emulsion of lecithin with the production of phosphocholine and a diglyceride. It seems probable therefore that the creaming of the yolk-emulsion is due to the decomposition of the lecithin present which acts as a stabilizing agent in the emulsion of protein and fat. The available evidence points to the fact that the lecithinase responsible for this action is identical with the enzyme responsible for the reaction on egg-yolk and human serum and therefore, from the work of Macfarlane *et al.* [1941], with the α-toxin.

Preparation of the toxins

Some details of the medium and the method of cultivation of the organisms may be recorded here as they have proved useful to other workers to whom they have been already communicated. The technique has been evolved in the Serum Department during a number of years, mainly by Dr D. W. Henderson. The medium described has given good yields of toxin (100–200 M.L.D./ml.), with the strain used, in a suitable condition for biochemical investigation. Variations of peptone and glucose concentration may be necessary for maximum yields of toxin with other strains.

The organisms were grown in a medium the final composition of which was essentially as follows:

(*a*)	3·3% Evans[1] peptone	800 ml.
(*b*)	Na_2SO_4 muscle-extract (see below)	100 ml.
(*c*)	0·5% glucose (added as a 4·5% solution in (*b*)).	

The Na_2SO_4 muscle-extract (*b*) is the only ingredient of the medium which calls for special comment. This was prepared from horse or beef muscle by the method of Deutsch *et al.* [1938], which yields a protein-free extract containing all the water-soluble constituents of the muscle including the more heat-labile materials. The constituents of this extract which appear to enhance toxin formation on this medium are being investigated.

Deutsch *et al.* used a small fruit-press warmed to 32° for expressing the juice from the warm mass of tissue and sulphate. For 10 kg. quantities of meat mince we have found it convenient to use 8–10 in. Büchner funnels attached to filter flasks and to apply a vacuum, the whole apparatus standing in a hot-room. A rubber sheet tied over the funnels by narrow rubber tubing is a con-

[1] Manufactured by Evans Sons, Lescher and Webb, Ltd., Runcorn, Cheshire.

siderable aid in expressing the juice. The layer of mince-sulphate sludge on a filter should be not more than 2 in. thick before sucking. The further stages of the preparation follow Deutsch *et al.* The extract usually has a *p*H of about 5; alkali is added, with stirring to avoid local excess, bringing the *p*H to 6·5. A more alkaline *p*H causes a precipitate and loss of potency for toxin production. We have been content with one extraction of the minced muscle, 10 kg. of mince yielding on an average 1500 ml. of extract.

The Na_2SO_4 muscle-extract must not be heated; it is sterilized by filtration and stored in the cold, after the required amount of glucose has been added to it.

Method of cultivation. The routine adopted was as follows:

1. 3·3% Evans peptone and 0·5% NaCl were dissolved in tap-water, adjusted to *p*H 7·6 with drops of 40% NaOH, warmed to flocculate the phosphate precipitate and filtered through paper (Chardin or Whatman No. 1). This peptone-water was dispensed in 800 ml. lots into 1000 ml. conical flasks, plugged, and autoclaved for 15 min. at 115°.

2. On the morning of inoculation the flasks of peptone-water were steamed for 30 min. and cooled rapidly to about 45°.

3. To each 800 ml. of the peptone-water were at once added 100 ml. of the cold sterile Na_2SO_4 muscle-extract containing 4·5% glucose. This was run in with aseptic precautions from a bulb pipette without unduly agitating the medium, to minimize re-oxygenation. Sometimes thiolacetic acid (10 ml. of 2 *M* thiolacetic acid in *N* HCl followed by an equivalent amount of 2 *N* NaOH) was added to facilitate the maintenance of anaerobic conditions suitable for the initiation of growth. This was however in general unnecessary under the conditions used and can be omitted.

4. The inoculum was from an 18 hr. culture in Robertson's meat medium of *Cl. welchii* (type A) strain 107 (N.C.T.C. No. 6125). About 5 ml. of the actively growing culture were carefully injected from a Pasteur pipette into the bottom layers of the medium. With this technique rapid initiation of growth was secured and there was no need for anaerobic incubation. The flasks were incubated at 34–35°. Gas evolution was vigorous and the frothing sometimes blew out the plugs. It was therefore convenient to place the flasks on trays which could afterwards be sterilized.

5. The cultures were incubated for 5–6 hr. only. The toxin concentration reached a peak between 5 and 6 hr. incubation, which represents approximately the end of the log phase of growth, under the conditions described here. After this time there was a considerable diminution in the amount of toxin present. A slackening in the rate of gas evolution was a rough index of the time at which to stop incubation.

6. The cultures were filtered at once through paper-pulp[1] 'puddings' formed on the outside of Berkefeld candles and held in place by wrappings of gauze tied with string.

Dried toxin preparations. Several preparations of dried toxin were made by 2/3 or full saturation of the filtrate with $(NH_4)_2SO_4$. A scum of crude toxic material rose to the surface and was rapidly skimmed off and placed on the top of piles of filter paper. Some fluid was removed by changing the underlying sheets. The top papers carrying the crude toxic material were then placed in a vacuum desiccator containing P_2O_5 and NaOH, and rapidly evacuated by a Hyvac pump. The P_2O_5 was changed once after about an hour and the desiccator re-evacuated. The product was a friable light solid easily powdered to a fine grey powder. The average yield was about 1 g. per l. of medium; more could have been recovered by filtration or more complete skimming, but yield was sacrificed for speed of manipulation at this stage.

Glycerinated toxic culture filtrates. Sterile filtrates were filled into cellophane tubing and dialysed against glycerol at 3°. The volume of culture filtrate within the tube decreased considerably as water diffused out into the glycerol, thus effecting a concentration, while glycerol more slowly diffused in. This yielded convenient stable preparations, both the M.L.D. and L+ values remaining constant over long periods when stored at 0°. One preparation has remained constant for over 9 months and shows no evidence of deterioration. This method [Vanicek, 1930] has proved to be useful for the preparation of stable test-toxins from toxic culture filtrates or autolysates of various organisms during the past ten years in the Serum Department of this Institute.

Biological potency. The minimum lethal dose of a toxin preparation was determined by the intravenous injection of the dose, in a total volume of 0·5 ml. in normal saline, into mice weighing

[1] T. B. Ford's 'plain filter pulp, cotton mixture without asbestos'. (Agents: Alfred K. Wright, 110 Saffron Hill, London, E.C. 1.)

17–20 g. A direct correlation between the M.L.D. and the content of a particular toxic component is not to be expected except in the absence of other toxic substances. The L+/5 dose of toxin is the least dose of toxin which when mixed with 1/5 unit of antitoxin and injected intravenously into 17–20 g. mice, killed some but not all of the group within 48 hr.

The M.L.D. of the toxin preparations used in the present work was as follows:

Dried toxin W.E. 6	0·008 mg.
Glycerinated toxin K 178–180	0·0025 ml.
Glycerinated toxin K 188	0·0022 ml.

The L+/5 dose of toxin K 178–180 was 0·025 ml.

Antitoxins. The ultimate reference antitoxin was the British Standard Gas Gangrene Antitoxin (*Perfringens*) containing 20 units/ml. This is an α-antitoxin standard. The working reference antitoxin was the Elstree Standard No. 1 containing 95 units/ml.

Biochemical action of *Cl. welchii* toxin

General methods. Total P was determined by preliminary ashing with perhydrol and H_2SO_4 and colorimetric estimation of the inorganic phosphate by a modification of Briggs' method [Martland & Robison, 1926]; the colour comparison was made with a Bausch and Lomb colorimeter against suitable standards ranging from 0·005 to 0·1 mg. P, with an accuracy of approximately ±0·0005 mg. P. Microestimations of N were made by the Kjeldahl method and of Ca by precipitation as oxalate and permanganate titration. The action of the toxins on egg-yolk was followed by the method devised by van Heyningen. The diluted toxin (approximately 1–3 M.L.D.) is allowed to act on the egg-yolk under standard conditions, the reaction stopped by addition of excess antitoxin and the turbidity measured in a colorimeter against that developed simultaneously by a known amount of toxin in the same conditions. We have used as a standard the glycerinated toxin K 178–180 and arbitrarily assigned to it a value of 200 egg-yolk units per ml.

Action of Cl. welchii *toxin on egg-yolk*

Decomposition of phospholipin. A sterile emulsion of one egg-yolk was prepared in 250 ml. of 0·9% NaCl. A sample of this emulsion was extracted three times with an equal volume of ether at room temperature, and the ethereal and aqueous solutions analysed. A second sample was treated with 2 mg. dry *Cl. welchii* toxin (W.E. 6) in presence of 0·01 *M* $CaCl_2$ for 4 hr. at 37° and analysed similarly. Table 2 shows that by the action of the toxin the original

Table 2. *Analysis of egg-yolk emulsion (a) before and (b) after the action of* Cl. welchii *toxin*

Values in mg./100 ml.

		Ether-soluble fraction			Protein		Acid-soluble fraction	
Total N	Total P	Wt.	N	P	N	P	N	P
(*a*) 43	12·0	850	4·0	5·3	34	5·5	5·0	1·2
(*b*) 43	12·0	750	0·2	0·2	31	0·5	9·0	11·5

lipin P was converted into an acid-soluble form; most of the P originally present in the protein component also became acid-soluble, although the protein N was only slightly decreased.

Comparison of rate of formation of acid-soluble P and development of turbidity. A number of tests were set up in duplicate in two series A and B as follows: 3·0 ml. diluted toxin (1/300) in 0·002 *M* $CaCl_2$; 1 ml. borate or acetate buffer;

1 ml. yolk emulsion, containing 0·12 mg. total P and 0·05 mg. lipin P per ml. After incubation at 37°, 5 ml. of a saline dilution of *Cl. welchii* antitoxic serum (10 units antitoxin) were added to the tube in series A to stop the reaction, and the relative turbidity estimated in the colorimeter against an arbitrary standard. 2 ml. 10 % trichloroacetic acid were added to the tubes in series B, and the amount of total P in the filtrate estimated; the amount of P liberated was calculated by subtraction from this figure of the original acid-soluble P present. Table 3, in which the relative degrees of hydrolysis and of turbidity are referred

Table 3. *Comparison of the rate of hydrolysis of P with development of turbidity in egg-yolk emulsion by* Cl. welchii *toxin*

		20 min.			35 min.		
			Relative degree of			Relative degree of	
Buffer	*p*H	mg. P hydrolysed	Hydrolysis	Turbidity	mg. P hydrolysed	Hydrolysis	Turbidity
Borate	7·1	0·031	1·0	1·0	0·041	1·3	1·4
Acetate	5·9	0·027	0·8	0·7	0·036	1·2	1·1
Borate	9·3	0·019	0·6	0·3	0·028	0·9	0·6

to the reaction at *p*H 7·1 for 20 min. as unity, shows that at *p*H 7·1 and 5·9 the rate of hydrolysis and the development of turbidity are almost parallel, while at *p*H 9·3 the hydrolysis is considerably faster than the increase in turbidity. It seems probable that the hydrolysis of the phospholipin is the primary action and that the aggregation of fat globules to which the turbidity is due is dependent on this hydrolysis but influenced by the physical conditions of the mixture.

Hydrolysis of egg lecithin by Cl. welchii *toxin*

Preparation of 'lecithin'. Three egg-yolks were washed, emulsified in 50 ml. 0·9 % NaCl and extracted 4–6 times with an equal volume of ether by shaking at room temperature and centrifuging. The ethereal solution was evaporated to 100 ml. and treated with 2 volumes of acetone; the precipitate was washed with acetone and taken up in 50 ml. ether; after centrifuging, the clear solution was again treated with acetone and the precipitate washed with acetone and dried in a vacuum desiccator over H_2SO_4. The product (1·5–2·0 g.) contained generally 3·7–3·9 % P and 1·9–2·1 % N and was completely soluble in ether; when freshly prepared it gave a stable emulsion with water which showed no change on standing at 0° for periods up to a week.

Course of hydrolysis. A typical hydrolysis was carried out as follows: 830 mg. lecithin (31 mg. P), emulsified in H_2O, 1·5 ml. 0·1 *M* $CaCl_2$ and 5 mg. dry *Cl. welchii* toxin (W.E. 6), were mixed in a total volume of 15 ml., adjusted to *p*H 7·4 with 0·1 *N* NaOH and placed in a thermostat at 37°; a further 2 mg. toxin were added after 5 hr. and the mixture was titrated back to *p*H 7·4 with 0·1 *N* NaOH at intervals until no further liberation of acid took place, by which time a layer of fat had risen to the surface. The fatty material was then removed from the aqueous portion by shaking 4 times with ether and separating the ethereal solution by centrifuging.

Identification of products. (*a*) *Phosphocholine.* Analysis of the aqueous solution showed that it contained practically all the original lipin P in an organic form, and an equivalent amount of N, but no free choline. A sample of the solution was treated at *p*H 8·5 with a bone phosphatase preparation free from diesterase; the organic P was rapidly and completely hydrolysed to inorganic phosphate, indicating that the compound was a monophosphoric ester, pre-

sumably of choline. The compound was slowly hydrolysed by *N* HCl at 100°, $k = 0{\cdot}15 \times 10^{-3}$; the hydrolysis curve agreed with that found by Plimmer & Burch [1937] for phosphocholine (phosphorylcholine), and the Florence test for choline was now positive in high dilutions.

The solution was evaporated to dryness and the organic P compound extracted from the salts with absolute alcohol. The alcoholic solution was evaporated to 16 ml. and 4 ml. 0·5 *M* $CaCl_2$ added. 186 mg. crystals were obtained in the first two crops, containing 70 % of the original lipin P; the composition of these crystals, analysed without recrystallization, agreed with that of the crystalline Ca compound of phosphocholine, $C_5H_{12}O_4NPClCa, 4H_2O$ [Plimmer & Burch, 1937]:

	% P	% N	% Cl	% Ca	% H_2O
Crop 1	9·75	4·1	10·2	12·2	18·5
Crop 2	8·7	3·5	11·8	10·7	22·3
Required	9·46	4·25	10·75	12·16	21·86

Without careful recrystallization this compound is apt to contain traces either of carbonate or of the acid salt.

(*b*) *Neutral fat.* The ethereal solution was evaporated to dryness and taken up in acetone leaving a small amount of acetone-insoluble material. The acetone-soluble material (574 mg.) was a neutral fat (acid value 1·1) with a saponification value of 172 (calc. for stearyloleylglyceride 176). The fatty acids were extracted from the saponified and acidified mixture with ether, and separated into saturated and unsaturated acids on the basis of the solubility of the Pb salts in 95 % alcohol. The nature of the fatty acids was not further determined, since it depends solely on the constitution of the original material. The aqueous portion of the saponification mixture was freed from H_2SO_4 with baryta and evaporated to dryness. The residue was extracted with an ether-alcohol (1 : 3) mixture which gave on evaporation 74 mg. syrup. The content of glycerol was estimated by refluxing with 2 ml. acetic anhydride and 0·5 g. Na acetate, and saponification of the resulting triacetin.

A balance sheet for two hydrolyses of this kind is shown in Table 4. The yields obtained are calculated on the basis that the whole of the lipin P was

Table 4. *Products of the hydrolysis of lecithin by* Cl. welchii *toxin*

	Exp. 1		Exp. 2	
mg. Lecithin	550		830	
mg. P	21·0		31·0	
Hydrolysis products:	Found	% of calc.	Found	% of calc.
(*a*) m.-mol. acid	0·73	108	0·93	93
(*b*) Water-soluble product:	mg.		mg.	
P	19·5	93	29	94
N	9·3	96	13	93
Phosphocholine	187	74	226	69
(*c*) Ether-soluble product:				
Neutral fat	350	82	574	92
(*d*) Saponification products of (*c*):				
Glycerol	46·4	74	68	74
Saturated fatty acids	131	68	233	82
Unsaturated fatty acids	148	77	294	104

present as a stearyloleyl derivative of glycerophosphocholine, since the substitution of other fatty acids makes no significant difference to the results. It will be seen that the data agree substantially with the decomposition of more

than 90 % of the original phospholipin, with liberation of one acidic group, into phosphocholine and a diglyceride containing one molecule each of glycerol, a saturated and an unsaturated fatty acid. The phospholipin used was not specifically free from kephalin or sphingomyelin. The P : N ratio and the yield of crystalline phosphocholine indicate that at least 70 % of the P was present as lecithin, but the possibility that the toxin decomposes other phospholipins and in particular kephalin is not excluded.

Characteristics of Cl. welchii *lecithinase*

The enzymic activity under various conditions was estimated by the rate of formation of acid-soluble P from an aqueous emulsion of lecithin by the glycerinated toxins K 178–180 and K 188, the general technique being as follows: 0·1 ml. toxin (approximately 40 M.L.D.) was diluted with water to 30·0 ml. and transferred to a stoppered 50 ml. conical flask, samples being removed with an Ostwald pipette. Uniform thin-walled test tubes were used and the test mixtures were made up with water to a total volume of 6 ml. including 1 ml. Palitzsch's borate buffer, *p*H 7·1; the tubes were placed in a thermostat at 37° and the reaction stopped by the addition of 1 ml. 20 % trichloroacetic acid. The solution was filtered from the flocculated lecithin within 15 min. through a No. 30 or 44 Whatman paper, and re-filtered if necessary; it is essential that the filtrate should be crystal clear to exclude the presence of unchanged lecithin. Total phosphate was determined in aliquots (usually 3 ml.) of the filtrate, the amounts being of the order 0·005–0·15 mg. P. It was found that no appreciable hydrolysis of lecithin took place in absence of toxin either at *p*H 7·1 at 37° or in trichloroacetic acid at room temperature for ½ hr. Blank values for the acid-soluble P contained in the toxin and lecithin were obtained by adding the trichloroacetic acid before the addition of lecithin.

Enzyme specificity. None of the toxins examined hydrolysed diphenyl-, monophenyl- or β-glycero-phosphate or nucleic acid; the enzyme is therefore a lecithinase, as distinct from a phosphodiesterase, although the linkage is of a diester type.

Stability. The concentrated glycerinated toxin preparations have remained unchanged in activity (M.L.D. and L+/5 value) for over 9 months when kept at 0°. Dilute solutions in saline used for these enzyme studies, containing 5 M.L.D./ml. or less, are stable at 18° for a few hours if kept with a small surface exposed to the air. The diluted enzyme is however very rapidly inactivated by the exposure of a shallow layer, by shaking or by bubbling air through it. This inactivation is very probably due to surface denaturation and not oxidation, since the enzyme was unaffected by treatment with a 1 : 100 dilution of '30 vol.' H_2O_2, while bubbling with N_2 which had been passed through alkaline $Na_2S_2O_4$ inactivated it.

In a bubbling experiment air was drawn uniformly through a dilute toxin solution so that good foaming occurred, and samples were withdrawn at intervals and tested for activity by the egg-yolk reaction and on mice. The mice were injected intravenously with a volume of diluted toxin calculated from the results of the egg-yolk reaction to contain just above and just below one M.L.D. The mouse deaths agreed reasonably well, n view of the few mice and levels of dose employed, with the expected toxicity The percentage inactivation of the toxin shown by the animal test (col. 4, Table 5) was calculated from these figures.

A similar more accurate test showed 35 ± 5 % inactivation of the lecithinase and 37 ± 10 % inactivation of the toxin as measured in mice. Because of the very great ease of surface denaturation it was essential to adopt uniform methods

Table 5. *Inactivation of lecithinase and toxin by bubbling*

Time of bubbling min.	M.L.D./ml. by egg-yolk reaction	Percentage of initial activity by	
		Egg-yolk reaction	Mouse test
0	4·5	100	(100)
15	3·8	84	100
30	2·8	62	—
40	2·7	60	66
60	1·8	45	<66 >25
90	0·8	17	—

for the dilution of the toxin and the addition of reagents, and to mix carefully without shaking, in order to get reproducible results with dilute solutions of the toxin.

The enzyme is comparatively heat-stable, 45 % of the activity remaining after heating in borate buffer *p*H 7·6 for 10 min. at 100° in a sealed ampoule; it is more rapidly inactivated in acid than in alkaline solution at this temperature (Table 6). It is possible that a large part of this apparent heat-inactivation is due to surface denaturation during the heating process.

Table 6. *Heat stability of lecithinase of toxin K 188 at 100°*

The toxin was heated at a dilution of 1 in 80 in the medium stated.

Medium	A water	B borate	C borate	D acetate
*p*H	*ca.* 7·0	7·6	7·1	6·0
	Percentage activity			
Time min.				
10	8	45	35	14
30	0	12	8	5

pH range. The enzyme is active over a wide *p*H range, with a broad optimum, 7·0–7·6, in borate buffer; the activity at *p*H 9·3 and 5·2 in borate and acetate buffers respectively was 64 and 66 % of the maximum. Acetate and veronal buffers have a slight and phosphate buffer a marked inhibiting effect compared with borate at the same *p*H.

Substrate concentration and activation by Ca^{++} ions. The activity of the enzyme is greatly affected by the presence of Ca^{++} ions, the activation being most marked with very small amounts of toxin or with low concentration of lecithin. The enzyme is also activated by low concentrations of Mg salts but inhibited in concentrations of 0·01 *M* or over. Table 7 shows the effect of variation in the

Table 7. *Effect of substrate concentration on the hydrolysis in 15 min. at 37° by 2·0 ml. 1/300 dilution of toxin K 188*

	Concentration of lecithin (*M*)				
	0·011	0·008	0·0053	0·0027	0·0011
Conc. of Ca^{++}	mg. acid-soluble P formed				
Nil	—	0·132	0·103	0·050	0·016
0·008 *M*	0·199	0·189	0·162	0·121	0·077

substrate concentration on the rate of hydrolysis with and without the addition of Ca. For the quantity of toxin used the velocity was almost maximal in 0·01 *M*

lecithin. Half this maximal velocity was reached at a substrate concentration of $0{\cdot}002\,M$ in presence of Ca, but only at $0{\cdot}005\,M$ in its absence; that is to say, the affinity for lecithin is more than twice as great in the presence of Ca.

Table 8 shows the activating effect of Ca^{++} on the hydrolysis of $0{\cdot}0005\,M$ lecithin under the same conditions as in Table 7. A concentration as low as $1{\cdot}6 \times 10^{-4} M$ Ca^{++} had a striking effect on the activity which was vanishingly

Table 8. *Effect of Ca^{++} on rate of hydrolysis*

1/300 dilution of toxin K 178–180 ml.	Conc. lecithin *M*	Conc. of added Ca^{++}, $\times 10^{-3} M$: Nil	0·16	0·32	0·8	1·6	2·4	3·2	8·0	16
		mg. acid-soluble P formed								
2·0	0·0005	0·005	0·026	0·033	0·035	0·040	0·045	—	—	—
3·0	0·0053	0·095	—	—	—	0·163	—	0·210	0·214	0·214
0·5	0·0053	0·011	—	—	—	0·022	—	0·027	0·036	0·037

small in the absence of added Ca^{++}. With higher substrate concentrations, in which an appreciable hydrolysis took place without the addition of Ca^{++}, the concentration of Ca^{++} needed to reach the maximum velocity varied with the amount of enzyme, being lowest with the highest amounts of enzyme (Table 8). It is possible that the partial activity of the enzyme without addition of Ca^{++} in high substrate concentrations was due to traces of Ca or Mg in the lecithin; analysis of one sample of lecithin showed that it contained less than 0·2 % Ca, but a content of 0·05 % of Ca or Mg would have an appreciable effect. The activity was practically maximal in $0{\cdot}01\,M$ $CaCl_2$ for amounts of enzyme between 0·5 and 3·0 M.L.D.

Examination of the hydrolysis curves (Table 9) shows that with low concentrations of toxin there is a marked induction period in the absence of Ca^{++} before any measurable hydrolysis takes place; this is reduced or abolished by

Table 9. *Effect of Ca^{++} on induction period of hydrolysis*

mg. P hydrolysed in total volume 25 ml. containing 1·9 mg. lecithin P.

ml. of 1/300 dilution of toxin K 178–180	5·0		1·0		0·5	
Conc. Ca^{++} (*M*)	Nil	0·008	Nil	0·008	Nil	0·008
Time (hr.)						
0·5	0·19	0·48	0·05	0·12	0·03	0·06
1·0	0·44	0·82	0·05	0·22	0·03	0·13
1·5	0·58	1·10	0·05	0·29	0·04	0·18
2·0	0·61	1·23	0·08	0·37	0·06	0·22
5·0	1·02	1·39	0·23	0·65	0·11	0·34
24	1·32	—	0·34	—	0·17	—

the addition of Ca^{++}. Since the velocity of the hydrolysis is also influenced by Ca^{++} and in any case falls off rapidly after a few hours, presumably owing to the gradual inactivation of the enzyme, the presence or absence of Ca^{++} has a profound effect on the total amount of hydrolysis by a given amount of toxin. The importance of this fact in estimations of the haemolytic activity of the toxin is discussed later.

Enzyme concentration. As a result of the foregoing experiments the following conditions were adopted for estimating the lecithinase activity: 0·2 ml. $0{\cdot}3\,M$ $CaCl_2$; 1 ml. borate buffer *p*H 7·1; toxin, any other reagent and H_2O to 5·0 ml.; 1 ml. 2·5 % aqueous lecithin. The reaction was stopped after 15 min. at 37° by the

addition of 1 ml. 20% trichloroacetic acid, and the total phosphate in the filtrate estimated as before, with the appropriate blanks. Table 10 shows that in these conditions the hydrolysis proceeds with almost linear velocity for 15 min. with the highest amount of toxin used, but falls off rapidly when more

Table 10. *Hydrolysis rate for 3·0 ml. of a 1/300 dilution of toxin K 178–180*

Total lecithin P = 1·0 mg.

Time min.	mg. P hydrolysed	Velocity constant $k \times 10^3$	% hydrolysis
10	0·140	7·4	15·7
15	0·195	7·0	21·4
20	0·237	6·6	26·3
30	0·309	6·1	34·2

Table 11. *Variation of hydrolysis with enzyme concentration*

1/300 dilution of toxin K 178–180	mg. P hydrolysed				
ml.	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean
3·0	0·198	0·213	0·207	0·180	0·198
2·0	0·140	0·148	0·147	0·125	0·140
1·0	0·073	0·076	0·065	0·069	0·071
0·5	0·031	0·036	—	0·033	0·033
0·2	0·008	—	0·009	0·016	—

than 20% of the substrate has been decomposed. Table 11 shows that the hydrolysis is proportional to the enzyme concentration when the actual hydrolysis is of the order 0·04–0·20 mg. P, and that the values for a given toxin are reproducible with an accuracy of approximately ±6%.

A lecithinase unit was arbitrarily defined as that quantity of enzyme which under the conditions prescribed above produced 0·1 mg. acid-soluble P from lecithin in 15 min. at 37°. The relationship between the minimum lethal dose, the egg-yolk unitage and the lecithinase activity of toxins K 178–180 and K 188 is shown in Table 12.

Table 12. *Titres of* Cl. welchii *toxins*

Toxin	Units/ml.		
	M.L.D.	Egg-yolk	Lecithinase
K 178–180	400	200	210
K 188	450	266	290
Ratio toxin $\frac{\text{K 178–180}}{\text{K 188}}$	0·89	0·75	0·73

Effect on lecithinase activity of other factors in Cl. welchii *toxins*

Toxoid. The toxoid prepared for the routine immunization of horses had no appreciable lecithinase activity in quantities equivalent to 15–20 lecithinase units of toxin. The activity of 2 lecithinase units of toxin was not significantly altered by the presence of an excess of toxoid.

β- and δ-toxins. We are indebted to Dr Oakley for a specimen of dry *Cl. welchii* type C toxin (NX 17) containing only traces of α-toxin; 1 ml. of a 1% solution of this toxin contained β-toxin equivalent to 55 β-units and δ-toxin equivalent to 1·85 δ-units. We found that a 1% solution contained 1·8 lecithinase units per ml.; a mixture of 0·5 ml. of this solution and 1·0 ml. of type A toxin hydrolysed 0·145 mg. P under standard conditions, the sum of the separate activities being 0·150 mg. P. There was therefore no significant inhibition of the lecithinase activity by the β-toxin and δ-toxin present.

Inhibition by Cl. welchii *antitoxic sera*

Measurement of inhibition. The inhibition of the lecithinase activity of the toxin by specific antitoxic sera was measured as follows: 2·0 ml. diluted toxin (1–2 units lecithinase) were allowed to react with a saline dilution of the serum in a total volume of 3·8 ml. for 15 min. at room temperature; 0·2 ml. 0·3 *M* $CaCl_2$, 1 ml. borate buffer *p*H 7·1 and 1 ml. 2·5 % lecithin were added and the amount of hydrolysis in 15 min. at 37° estimated in the usual way. A simultaneous estimation of the hydrolysis by the same quantity of toxin in conditions identical except for the omission of serum was always carried out. Table 13

Table 13. *Inhibition of varying amounts of lecithinase by antitoxin*

15 min. hydrolysis at 37°.

Toxin (1/300 dilution)	ml.	mg. P hydrolysed without antitoxin	Decrease in P hydrolysed with antitoxin: 0·01 unit mg.	0·01 unit %	0·03 unit mg.	0·03 unit %	0·05 unit mg.	0·05 unit %	0·10 unit mg.	0·10 unit %
K 178–180	3·0	0·239	0·018	7	0·068	29	0·133	56	0·205	86
,,	2·0	0·148	0·012	8	0·039	26	0·092	62	0·129	87
,,	1·0	0·076	0·011	14	0·025	33	0·046	60	0·068	89
K 188	2·0	0·205	0·021	10	0·077	38	0·134	65	0·192	94

shows that the combination of the enzyme with the anti-lecithinase does not take place by simple multiple proportions, the absolute decrease in the hydrolysis with a given amount of serum decreasing with decreasing concentration of enzyme. It will be seen that in this experiment the percentage inhibition of the activity by a given amount of serum is relatively almost independent of the enzyme concentration in the limited range tested, considering the experimental variation in tests made at different times. Slight variations in the absolute amount of hydrolysis due to inactivation of the enzyme or variation in the time or temperature of the reaction therefore do not appreciably affect the results if these are calculated as percentages of the uninhibited hydrolysis carried out simultaneously.

Titration of antisera. The experiments in Table 13 were carried out with the Elstree No. 1 standard antitoxin (*Cl. welchii* type A) containing 95 international units/ml. This serum has been taken as a standard and arbitrarily defined as containing 95 anti-lecithinase units/ml. Table 14 shows the percentage inhibition

Table 14. *Inhibition of lecithinase by antitoxin*

Antitoxin units	% inhibition	Mean	Antitoxin units	% inhibition	Mean
0·005	4, 8, 4	5	0·06	81, 74, 77, 77	77
0·01	10, 17, 11	13	0·07	85, 76, 84, 84	82
0·02	32, 24, 29, 29, 25	28	0·08	92, 82, 85, 86	86
0·03	46, 35, 44, 37, 38	40	0·09	96, 88, 94, 91	92
0·04	49, 52, 51, 48	50	0·10	94, 96	95
0·05	70, 72, 68, 65, 67	68	—	—	—

of the hydrolysis by 1·9 lecithinase units of toxin K 188 produced by amounts of this serum containing from 0·01 to 0·1 antitoxin units. From these values a curve was constructed, plotting the percentage inhibition against the number of anti-lecithinase units. The concentration of anti-lecithinase in other sera was found with an accuracy of approximately ± 10 % by reference to this curve, after

estimation of the percentage inhibition of this toxin produced by 2 or 3 different quantities of the unknown serum in the conditions prescribed above. It was usually necessary to make at least 1/1000 dilution of the serum, so that the amount of phosphate added in the serum was negligible in these cases. The lecithinase activity was not significantly altered by the addition of normal or diphtheria antitoxic horse sera in quantities 10 to 50 times greater than those of the *Cl. welchii* antitoxic sera used.

Table 15 shows the antitoxic units per ml. of a number of *Cl. welchii* antitoxic horse sera determined by different methods. It was found that the titres of the type A sera determined by the anti-lecithinase method ran parallel with the

Table 15. *Titres of* Cl. welchii *antitoxic horse sera*

	Antitoxin units/ml.		
Serum	L+ method*	Egg yolk	Anti-lecithinase
Type A antisera:			
Elstree Standard No. 1	95	95	95
'Aden'	550	500	600
'Berbera'	350	340	330
'Crete'	—	960	1000
'Eritrea'	350	240	420
'Massawa'	200	170	260
'Sollum'	530	400	440
Type C antisera	α-Antitoxin		
R 7157	85	—	80
R 7164	48	—	62

* The animal test values for the type A sera are taken from routine testing results which were done at 100 unit intervals. The measurements were not designed to test accurately the correspondence between the animal and *in vitro* tests.

egg-yolk titres; the latter have already been shown by Nagler [1939] and by Macfarlane *et al.* [1941] to run parallel with those obtained by the animal protection (L+) method. There seems little doubt that the two *in vitro* tests measure the α-antitoxin and for convenience the unitage of the standard serum Elstree No. 1 by the egg-yolk and lecithinase titrations has been arbitrarily fixed at the same numerical value as that found by the L+ method, i.e. 95 units/ml.

The sera R. 7157 and R. 7164, for which we are indebted to Dr Oakley, are type C sera with the following antitoxin units/ml.; α, 85 and 48; β, 3000 and 1000; δ, 0·5 and 80 respectively. Table 15 shows that the anti-lecithinase titres found by us agree well with the α-antitoxin titres obtained by a different method in a different laboratory. It seems probable that the anti-lecithinase titre is not affected by excess of β-antitoxin, and only slightly if at all by excess of δ-antitoxin.

Effect of Ca^{++} on the inhibition of lecithinase by antitoxic sera. When the lecithinase activity is measured in the presence of Ca^{++}, a larger amount of antitoxin is required for the neutralization (100% inhibition) of a given amount of toxin than in absence of Ca^{++} (Table 16). Taking the formation of 0·005 mg. acid-soluble P in 15 min. as the lowest limit of appreciable hydrolysis (analogous with the estimation of a minimum haemolytic dose) the decrease in the hydrolysis to this amount in this experiment required the addition of only 0·1 unit of antitoxin in absence of Ca^{++} but of 0·19 unit in the presence of Ca^{++}. The regulation of the Ca^{++} concentration is therefore of great importance in any *in vitro* tests of the activity of the α-toxin or of α-antitoxins, including the haemolytic titre. Table 17 shows the effect of Ca^{++} on the minimum haemolytic

Table 16. *Effect of Ca^{++} on inhibition by antitoxin*

mg. P hydrolysed by 3·0 ml. toxin K 178–180 (1/300) in 15 min.

Conc. Ca^{++}	Antitoxin units								
M	Nil	0·01	0·02	0·03	0·04	0·05	0·10	0·15	0·20
Nil	0·110	0·096	0·081	0·065	0·052	0·030	0·001	Nil	Nil
0·01	0·239	0·221	0·197	0·171	0·136	0·106	0·034	0·007	0·005

Table 17. *Effect of Ca^{++} on haemolytic titre of toxin K 178–180*

Diluent	ml. toxin	
	M.H.D.	$L_h/5$ dose
'Analytical Reagent' NaCl	0·0006	0·080
'Analytical Reagent' NaCl + *M*/100 Ca^{++}	0·0002	0·069
'Commercial Pure' NaCl	0·0002	—

dose and on the $L_h/5$ dose of toxin K 178–180. It will be seen that the substitution of C.P. for A.R. sodium chloride in the saline used for dilution is sufficient to reduce the M.H.D. from 0·0006 to 0·0002 ml. presumably through addition of Ca^{++} or Mg^{++}, and that the $L_h/5$ dose of toxin was reduced from 0·08 to 0·07 ml. in presence of 0·01 *M* $CaCl_2$. The values in these particular tests do not necessarily give the full haemolytic titre, since no attempt was made to secure optimal conditions for other haemolysins present, but they confirm that one haemolysin present, presumably the α-toxin, is affected by Ca^{++}.

It was shown by Neill [1926] that a reversibly reducible haemolysin was present in *Cl. welchii* culture filtrates of which only the reduced form was haemolytic. It seems probable that the considerable variation in the M.H.D. which has been found on occasion in different laboratories for the same toxin [cf. Ipsen *et al.* 1939] may be due in part to variation in the Ca^{++} concentration and in part to the degree of oxidation of the reversibly reducible haemolysin in the different tests.

Combining power of toxin with antitoxin. An attempt was made to assess the combining power of the toxin with antitoxin, as in the estimation of the L+ dose of toxin, by finding the smallest amount of toxin which, after combination with 0·2 unit of antitoxin, still hydrolysed lecithin at a rate approximately equivalent to 1·0 M.L.D. of toxin; this rate was taken as a hydrolysis of 0·05 mg. P in 15 min. at 37°. The estimation of this dose is materially affected by the dilution at which the enzyme action is allowed to proceed (Table 18). The toxin

Table 18. *Lecithinase activity in presence of 0·2 unit antitoxin*

Volume during hydrolysis	ml. toxin K 188				
	0·032	0·028	0·024	0·020	0·016
	mg. P hydrolysed in 15 min.				
A 4 ml.	0·039	0·021	0·008	0·007	0·004
B 20 ml.	0·065	0·032	0·022	0·007	0·007

and antitoxin were allowed to combine for 15 min. at room temperature in a total volume of 2·4 ml.; the hydrolysis was then carried out in a final volume of 4 ml. in series A and 20 ml. in series B, the volumes of $CaCl_2$, borate buffer and lecithin being adjusted to give the same concentrations in both series. It will be seen that the amount of toxin required is greater in the smaller volume, the actual difference of approximately 0·003 ml. of toxin representing in this

case about $1\frac{1}{2}$ lethal doses. It seems probable that the increased lecithinase activity on dilution is due to dissociation of the toxin-antitoxin complex; it remains to be seen whether other differences in sera or serum fractions can be detected by the reaction.

Inhibition by non-specific agents

Calcium precipitants. The enzymic activity is reduced by the presence of fluoride, citrate and phosphate. The concentration of these salts necessary to produce complete inhibition is dependent on the Ca^{++} concentration in the test mixtures; it appears that the inhibition is due to the reduction of Ca^{++} below a certain threshold value.

Antiseptics etc. The effects of a number of other substances are shown in Table 19; the substances were carefully mixed with the diluted toxin and allowed to stand 10 min. at room temperature before the addition of lecithin in the usual test.

Table 19. *Effect of various substances on lecithinase activity*

Substance	Conc. %	Enzyme activity %	Substance	Conc. %	Enzyme activity %
m-Cresol	0·25	150	Eserine	0·15	95
,,	0·025	130	Prostigmine	0·04	100
Euflavine	0·1	80	Urea	2·5	95
Proflavine	0·1	78	Na dodecyl sulphate	0·25	0
Sulphanilamide	0·2	97	,,	0·03	5
,,	0·025	100	Na oleate	0·08	50
Sulphapyridine	0·05	100	Na taurocholate	0·08	90
Glycerol	0·1	100	Digitonin	0·25	60

Sulphanilamide and sulphapyridine in final concentration of 0·2 and 0·05 % respectively had no appreciable effect, while only a slight inhibition was produced by 0·1 % of proflavine or euflavine, and the activity was actually increased in presence of *m*-cresol. Prostigmine and physostigmine (eserine) which inhibit the action of choline esterase in high dilution had no effect on the lecithinase in a final concentration of 0·04 %. The most effective inhibiting agent was Na dodecyl sulphate with which inhibitions of 80–100 % were obtained in concentrations as low as 0·025 %.

Discussion

The enzymic decomposition of lecithin into phosphocholine and a diglyceride has not previously been well established. According to Udagowa [1935] an enzyme of this type is present in takadiastase but the reaction products were not isolated. In the present case the absence of phosphomono- and di-esterase and lipases from the toxin enabled the quantitative isolation of the reaction products to be made without difficulty. The lecithinase present in snake venoms, which decomposes lecithin into an unsaturated fatty acid and lysolecithin, was designated by Belfanti *et al.* [1936] as 'Lecithase A'; that present in tissues, rice bran and other sources, which splits off both fatty acids from lecithin, was classified by these authors as 'Lecithase B'. The enzyme in *Cl. welchii* toxins, which decomposes lecithin with formation of phosphocholine and a diglyceride may, therefore, be termed a lecithinase C. Like the other lecithinases and the simple lipases, this enzyme is activated by Ca^{++} ions. Apart from its distinction from phosphodiesterase, its specificity has not yet been investigated.

From the pathological point of view the chief interest of the lecithinase in *Cl. welchii* toxins is its probable identity with the α-toxin, the lethal, haemolytic

and necrotic factor of this toxin. It has been shown by Nagler [1939] and Macfarlane *et al.* [1941] that the activity of the toxin in producing an opalescence in human serum or egg-yolk is a fair measure of the content of α-toxin. We have found that the development of opalescence is accompanied, and in certain circumstances preceded, by the decomposition of the lecithin present, and that like the former phenomenon this decomposition is activated by Ca^{++} and inhibited by citrate, fluoride and phosphate. The lecithinase of the toxin is inhibited by specific antitoxic sera, the inhibitory action running parallel in various type A antisera with the protective power of the serum *in vivo*, while in two type C sera the antilecithinase activity was in good agreement with an independent assay of the α-antitoxin content. The evidence is, therefore, strongly suggestive of the identity of this lecithinase with the α-toxin, though the ultimate proof of this is dependent on the isolation of a homogeneous substance with the requisite biochemical and pathogenic properties. It is, of course, inherently probable that this lecithinase would confer haemolytic and necrotic powers on the toxin, since it would have a disintegrating effect on any cell membrane or intracellular lipoprotein complex whose integrity depended on the presence of lecithin. Moreover, the lecithinase activity of the toxins examined is so high that it may well account for the pathological effect; an amount roughly equivalent to one lethal dose for a mouse could at its maximum velocity hydrolyse the whole of the blood lecithin of the animal in 2 or 3 hr. It seems possible that the inhibitory action of this toxin on the aerobic oxidation of succinate observed by Wooldridge & Higginbottom [1938] may have been due to a disintegration of cell structure leading to a disorientation of the various enzyme systems concerned rather than to a specific inhibition of any one enzyme.

Though the parallelism between the lethal dosage, the egg-yolk unitage and the lecithinase activity in the type A toxins examined was sufficiently close to indicate that the latter activity corresponded with the predominant lethal toxin, it was not quite so close as would be expected if the lecithinase were the sole agent in the biological effect. There are, however, two other factors at least which must be taken into account, hyaluronidase [McClean & Hale, 1941] and the second haemolytic factor, θ-toxin [see Macfarlane *et al.* 1941]. The contributions of all these various factors to the biological effect can hardly be gauged until they are obtained free from each other. Nevertheless the lecithinase activity of type A toxins is a guide to their biological potency which can at least effect a considerable economy in animal tests.

The regulation of the calcium concentration is of great importance both in the estimation of lecithinase and in that of the haemolytic titre of the toxin. It has been shown in this paper that the effect of Ca^{++} is to increase the affinity of the enzyme for lecithin and that the effect on the amount of lecithin hydrolysed is very marked when the concentrations of toxin and lecithin are low, as is the case in haemolytic tests. If, as is probable, the lecithinase of *Cl. welchii* toxins is haemolytic the mechanism of haemolysis is in interesting contrast to that of the lecithinase of snake venom. In the latter case, the phosphoric acid group of the decomposed lecithin is still present as a lipin, lysolecithin, which is in itself a highly haemolytic substance. The decomposition of lecithin by *Cl. welchii* toxin constitutes a more drastic change in physical properties, since the phosphoric acid group is now present as a water-soluble compound, but the products of the decomposition are innocuous. The pharmacological action of phosphocholine has been shown [Beznák & Chain, 1937] to be surprisingly weak, less than that of choline, and it seems improbable that its formation has any secondary consequences in the pathology of *Cl. welchii* infections.

Most of the bactericidal and other drugs examined had little effect in concentrations of less than 1 % on the lecithinase activity of the toxin. The failure to obtain any inhibition of the lecithinase by sulphanilamide may be specially mentioned; this is in agreement with the findings of Osgood & Powell [1938] but contrary to those of Carpenter & Barbour [1939] who found that this drug had a protective effect in a concentration of one in a million. The most effective inhibitory agent found was Na dodecyl sulphate and in view of the ease with which the toxin is inactivated by surface denaturation, it will be of interest to examine the properties of the toxin by surface film techniques.

The estimation of the lecithinase activity of a toxin has the advantage that the results can be expressed in absolute units independent of any particular toxin, but since it involves a microestimation of total P it is more laborious than the measurement of relative turbidities necessary in the egg-yolk technique devised by van Heyningen. The latter is on this account preferable as a rapid routine method of the assay of this activity in toxic culture filtrates by direct comparison with a toxin of known activity. The absolute amount of turbidity developed by a stable toxin however was found to vary considerably with different emulsions of egg-yolk and from day to day with the same egg-yolk emulsion. This difficulty was doubtless due in part to variation in the substrate concentration and could be largely overcome by diluting the emulsions of egg-yolk to a constant content of phospholipin and adding the optimum amount of Ca^{++}. The emulsions however tended to become opalescent on standing at 0° even if sterile, and this progressive change appeared particularly to affect the antitoxin titrations, so that it was necessary to compare the inhibitory effect of the unknown sera with that of a standard antitoxin in every separate test. The titration of antitoxic sera by estimation of the anti-lecithinase activity for routine purposes is not numerically more accurate than the egg-yolk test, since the error due to variations in enzymic activity in individual tests in both cases is greater than that of the estimation of P or of turbidity; it is preferable in that it is based on a specific reaction with a purified substrate, and should be a convenient method for investigation of the toxin-antitoxin reaction.

Summary

1. *Cl. welchii* (type A) toxin contains a lecithinase (optimum *p*H 7·0–7·6) which decomposes lecithin into phosphocholine and a diglyceride.

2. This lecithinase is probably identical with the specific α-toxin which is the lethal, haemolytic and necrotic substance predominant in type A culture filtrates.

3. The lecithinase is relatively heat-stable but is readily inactivated by surface denaturation and by sodium dodecyl sulphate. The enzyme is activated by Ca^{++} and inhibited by fluoride, citrate and phosphate, and is specifically inhibited by *Cl. welchii* (type A) antitoxic sera.

4. Methods are described for the estimation of the lecithinase activity of toxins and the corresponding anti-lecithinase activity of *Cl. welchii* antitoxic sera. The anti-lecithinase activity of *Cl. welchii* (type A) antitoxic sera runs parallel with the animal protection titre (L+) and is a fair measure of the α-antitoxin.

5. The rate and amount of hydrolysis by the lecithinase, alone and in the presence of antitoxin, are greatly affected by the concentration of Ca^{++} present. The significance of these results is discussed in relation to the estimation of the haemolytic titre of *Cl. welchii* toxins.

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99. THE DEGRADATION OF MUCINS AND POLYSACCHARIDES BY ASCORBIC ACID AND HYDROGEN PEROXIDE[1,2]

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It has been demonstrated [Robertson *et al.* 1940] that fresh vitreous humour of steers contains a labile substance capable of reducing the viscosity of synovial fluid mucin. The fact that the vitreous humour lost this activity upon standing, upon dialysis or upon addition of oxidizing agents, and the high vitamin C content of vitreous humour (approximately 0·5 mg./ml. as determined by 2:6-dichlorophenolindophenol titration), suggested that ascorbic acid might be an important factor in the breakdown of mucin.

This hypothesis was supported by the results of an experiment in which 100 mg. of ascorbic acid added to 25 mg. of mucin in *M*/20 phosphate buffer *p*H 7·2 caused a degradation of mucin in 2 hr., as shown by a reduction of the relative viscosity from 40 to *ca.* 1. Upon addition of acetic acid, a flocculent precipitate was obtained instead of the characteristic cohesive, fibrous precipitate of normal mucin. That the reduction in viscosity was irreversible, due to a change in the size of the mucoprotein molecule, and not caused by altered characteristics of the solvent, was proved by redissolving the precipitate in phosphate buffer at *p*H 7·2. The degraded mucin did not produce a viscous solution as does the fibrous precipitate of normal mucin. Reduction in the size of the mucin molecule was further shown by the fact that the degraded mucin passed through a Seitz filter (EK 6) which held back normal mucin. The characteristics of the altered mucin indicated that ascorbic acid had split the original macromolecules. This breakdown may or may not have been due to hydrolysis. However, the absence of free amino-sugars or of an increased concentration of reducing substances, even after a prolonged reaction period, demonstrated that there was no further hydrolysis.

The present investigation was undertaken to determine the role of ascorbic acid in the breakdown of mucin and to establish the degree of specificity of the reaction.

Methods

Ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol in HPO_3. H_2O_2 and traces of Cu salts, one or both of which were present in some of the ascorbic acid solutions, did not affect the determinations.

[1] This is publication No. 56 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School, Boston, Massachusetts.

[2] The expenses of this investigation have been defrayed by a grant from the Commonwealth Fund.

H_2O_2 was determined by titration with $KMnO_4$ in acid solution. When H_2O_2 was to be determined in the presence of ascorbic acid, which also reduces $KMnO_4$, titrations were made before and after the addition of catalase and the H_2O_2 calculated by difference.

Phosphate was determined according to Tschopp & Tschopp [1932].

The method for determining the time required for degradation of mucin was based on the change in the type of precipitate obtained with acetic acid [Robertson *et al.* 1940]. Portions of the reacting ascorbic acid-mucin mixture were added, at frequent intervals, to dilute acetic acid until the resulting precipitate was flocculent.

The role of ascorbic acid in the degradation of mucin

In an attempt to determine whether reduced or oxidized ascorbic acid is active in the degradation of mucin, the two primary oxidation products were tested. Neither dehydroascorbic acid[1] nor the irreversible oxidation product formed by opening of the lactone ring[2] [Herbert *et al.* 1933] had any effect on mucin. However, the time of breakdown of mucin by ascorbic acid could be decreased some 250-fold by addition of dilute H_2O_2. The degradation was not due to a change in the *p*H of the buffered mucin solution, and H_2O_2, when present alone, caused no change in mucin.

By studying the activity, in the presence of H_2O_2, of ascorbic acid oxidized by $K_3Fe(CN)_6$, it was demonstrated that equimolar concentrations of ascorbic acid and dehydroascorbic acid together cause no degradation, and that dehydroascorbic acid even in the presence of H_2O_2 is not capable of splitting mucin. This inactivity is not due to inhibition by $K_4Fe(CN)_6$.

The above results suggested that reduced ascorbic acid and H_2O_2 are both essential for the breakdown of mucin. Corroboration was obtained by experiments in which ascorbic acid, partially autoxidized in the presence of Cu, was the active agent. The activation in this case is presumably due to the presence of H_2O_2, which according to Barron *et al.* [1935] is formed during the autoxidation. 5 mg. of ascorbic acid in 1 ml. $M/100$ $CuSO_4$ added to 3 ml. (15 mg.) of mucin solution caused a breakdown in 8 min. at *p*H 7·2 and 30°. A similar solution of ascorbic acid containing catalase (crystalline preparation [Sumner & Dounce, 1937]) produced no degradation. The addition of catalase, which destroys the H_2O_2 as it is formed, completely prevented the degradation of mucin, demonstrating the indispensability of H_2O_2 in this reaction.

In order to test the specificity of this action with respect to ascorbic acid, several organic reductants, some of which contained the ene-diol grouping, were substituted for ascorbic acid. *d-iso*Ascorbic acid,[3] acetone ascorbic acid[3] and *l*-saccharogulonoascorbic acid[3] were equally active. Dihydroxymaleic acid, reductic acid, reductone and pyrogallol were less efficacious, having activities which decreased in the order named. Monomethyl ascorbic acid, diacetone gulosonic acid,[4] catechol, adrenaline, glutathione, cysteine, glucose, oxalic acid and formaldehyde were inactive.

[1] Dehydroascorbic acid was formed by the oxidation of ascorbic acid at *p*H 5·0 with $K_3Fe(CN)_6$. At *p*H 5·0 and 28° dehydroascorbic acid has a half life of about 3 hr. [Ball, 1937].

[2] Irreversibly oxidized ascorbic acid was formed by aerating ascorbic acid overnight in the presence of traces of Cu^{++} at *p*H 7·4 and 38°.

[3] We wish to thank Merck and Company for supplying these products.

[4] We wish to thank Hoffmann-La Roche for supplying the diacetone gulosonic acid and the ascorbic acid used in this investigation.

In an attempt to clarify the synergistic action of ene-diols, such as ascorbic acid, and H_2O_2 in causing degradation of mucin, the oxidation of ascorbic acid by H_2O_2 was studied.

Ascorbic acid and H_2O_2 react mol. for mol. as is clearly shown by Table 1, which gives the results of an experiment in which the molar ratio of H_2O_2 to ascorbic acid was varied from 0 to 4.

Table 1. *Oxidation of ascorbic acid by H_2O_2*

	0.00 hr. m.equiv. present		50.00 hr. m.equiv. present		m.equiv. reacted	
Experiment	H_2O_2	Ascorbic acid	H_2O_2	Ascorbic acid	H_2O_2	Ascorbic acid
A	0·00	0·56	0·00	0·53	0·00	0·03
B	0·13	0·56	0·00	0·44	0·13	0·12
C	0·25	0·56	0·00	0·28	0·25	0·28
D	0·50	0·56	0·03	0·13	0·47	0·43
E	1·00	0·56	0·30	0·06	0·70	0·50
F	2·00	0·56	1·50	0·06	0·50	0·50

The H_2O_2 and ascorbic acid were dissolved in 40 ml. and kept under oil at 34°. No attempt was made to remove traces of heavy metals.

The time required for degradation of mucin was shortened by increasing the concentration of ascorbic acid in the presence of excess H_2O_2 (Table 2). The change in rate, however, was not directly proportional to the increase in ascorbic acid concentration. Similarly a change in H_2O_2 concentration in the presence of a constant amount of ascorbic acid did not affect the rate of breakdown proportionately (Table 2).

Table 2. *Effects of ascorbic acid and H_2O_2 concentrations on the time of degradation of mucin*

Exp.	H_2O_2 mg.	Ascorbic acid mg.	Time for breakdown of 15 mg. mucin at *p*H 7·2 and 30° min.
1	30	0·156	55
		0·312	25
		0·625	15
		1·25	12
		2·5	8
		5·0	5
2	60	0·156	84
		0·312	35
		0·625	17
		1·25	11
		2·5	6½
		5·0	4
3	0	2·5	>120
	0·13	—	9
	0·65	—	5½
	7·8	—	3
4	0	2·5	>120
	0·13	—	9
	0·26	—	7
	0·52	—	5
	0·78	—	4

The requisite amounts of H_2O_2 and ascorbic acid were dissolved in water and 1 ml. of the solution was added to 3 ml. of the mucin solution.

Cysteine and cyanide which inhibit the autoxidation of ascorbic acid are not inhibitors of the degradation caused by ascorbic acid and H_2O_2. Neither appreciably affects the rate of breakdown of mucin.

No constant temperature coefficient for the degradation of mucin could be obtained. It varied with temperature and with the concentration of H_2O_2 and ascorbic acid. The measured coefficient was a function of at least two reactions, the change in the macromolecule and the oxidation of ascorbic acid. At temperatures above 60° the oxidation of ascorbic acid predominated so that degradation was complete only when high concentrations of ascorbic acid were used.

Similarly the effect of hydrion concentration on the degradation was the resultant effect on the aforementioned reactions. However, in this case the data indicated a consistent trend: the activity diminished rapidly as the *p*H increased, as illustrated by the following experiment.

1 ml. 0·005 *M* ascorbic acid in 0·25 % H_2O_2 was added to 3 ml. mucin solution (15 mg.) in *M*/20 phosphate buffers of different hydrion concentrations from *p*H 5·35–8. At acidities greater than *p*H 5, mucin precipitates, thus precluding an extension of these experiments to a more acid range.

*p*H (glass electrode)	5·35	5·9	6·4	6·85	7·2	8·1
Rate of mucin degradation mg./min. (at 30°)	3·55	2·5	0·96	0·62	0·3	0·06

Specificity of the ascorbic acid-H_2O_2 system

The action of ascorbic acid was tested on mucins of mesothelial and epithelial origin, on several viscous polysaccharides, on pneumococci and on β-glycerophosphate.

(*a*) *Mucins of mesothelial origin.* Degradation of a mesothelial mucin was demonstrated in the above experiments in which synovial mucin was the substrate. Ascorbic acid plus H_2O_2 caused degradation of other mesothelial mucins (umbilical cord and connective tissue [Robertson *et al.* 1940]) and of vitreous humour, with resulting loss of viscosity and change in the characteristic acetic acid precipitability.

(*b*) *Mucins of epithelial origin.* Ascorbic acid and H_2O_2 caused a similar alteration of the viscosity and precipitability of epithelial mucins (salivary and gastric) as shown by the following experiments.

10 g. commercial gastric mucin (Wilson) were shaken overnight with 100 ml. *M*/15 Na_2HPO_4 and then centrifuged. To 10 ml. of mucin solution were added 1 ml. 1 % H_2O_2 and 20 mg. ascorbic acid. The relative viscosity of 85·6 at 5 min. was reduced to 13·4 after 30 min. and to 5·3 after 16 hr.

Salivary mucin was prepared by precipitating saliva with 2 volumes of alcohol and redissolving the mucin in 0·2 volume of phosphate buffer *p*H 7·2. To 10 ml. of this mucin solution were added 20 mg. ascorbic acid in 1 ml. 1 % H_2O_2. The relative viscosity after 1 hr. was 1·61 in contrast to 2·05 for the control containing 1 ml. H_2O_2. Upon precipitation with alcohol, the control yielded the typical ropey precipitate, whereas the precipitate of the digest was finely flocculent.

(*c*) *Polysaccharides.* Because of the apparent lack of specificity of the ascorbic acid-H_2O_2 system, the action was tested also on starch paste, pectin, flaxseed mucilage, the polysaccharide of synovial mucin and chondroitin sulphuric acid from cartilage. There was a marked reduction in viscosity and change in characteristic precipitability when present.

Potato starch was dissolved in hot phosphate buffer. To 10 ml. of the cooled starch paste 1 ml. 1 % H_2O_2 and 20 mg. ascorbic acid were added. The relative viscosity after 1 hr. was 4·05 in contrast to 497 for the control containing 1 ml. H_2O_2. The iodine reaction remained unchanged after 24 hr.

A pectin solution (Certo) was dialysed against $M/20$ Na_2HPO_4. The *p*H was 6·2. 3 ml. $M/5$ buffer *p*H 6·5 containing 100 mg. ascorbic acid and 1% H_2O_2 were added to 10 ml. of the pectin solution. The relative viscosity after 10 min. was 2·3 compared with 22·4 for the control containing 3 ml. H_2O_2.

Flaxseed mucilage prepared by alcoholic precipitation of an aqueous extract of flaxseed was dissolved in phosphate buffer *p*H 7·2. 1 ml. of ascorbic acid (5 mg.) in 1% H_2O_2 was added to 10 ml. of this solution. After 10 min. the relative viscosity at 38° was 1·22 in contrast to 5·45 for the control solution containing 1 ml. 1% H_2O_2.

The polysaccharide prepared by tryptic digestion of synovial mucin [Robertson *et al.* 1940] was dissolved in phosphate buffer. 1 ml. 1% H_2O_2 containing 20 mg. of ascorbic acid was added to 10 ml. of polysaccharide solution. After 15 min. the relative viscosity was 1·25 in contrast to 59·6 for the control containing H_2O_2.

Two samples of chondroitin sulphuric acid from xyphoid cartilage were studied. One was prepared by 10% $CaCl_2$ extraction according to Meyer & Smyth [1937], the other by 2% KOH extraction. 1 ml. 1% H_2O_2 containing 20 mg. ascorbic acid was added to 5 ml. of chondroitin sulphuric acid solution buffered at *p*H 6·8. The controls contained H_2O_2 instead of ascorbic acid plus H_2O_2. The results with both samples were essentially the same, the relative viscosity after 1 hr. averaging 2·9 in contrast to 6·9 for the controls.

(*d*) *Pneumococci.* In view of the polysaccharide content of the pneumococcus capsule, the action of ascorbic acid and H_2O_2 on pneumococci was studied.[1]

Suspensions of heat-killed encapsulated pneumococci were prepared according to the method of Dubos [1937]. 0·5 ml. of this suspension was pipetted into precipitin reaction tubes and one drop of chloroform was added. 0·25 ml. each of buffered ascorbic acid, H_2O_2 and/or water was added and the tubes were incubated overnight. Microscopic examination of film preparations of each culture showed destruction of the capsule only in those cases in which both ascorbic acid and H_2O_2 were present, as shown below. The *p*H remained approximately 7 in all cases.

	Microscopic examination of pneumococci					
Type	I*	II	III*	VIII	XII	XVIII
Ascorbic acid and H_2O_2	Lysis	Lysis	Lysis	Lysis	Lysis	Lysis
Ascorbic acid (5 mg.)			Gram stain positive			
3% H_2O_2			Gram stain positive			
Water			Gram stain positive			

* Two different cultures of types I and III were tested.

(*e*) *Dephosphorylation of β-glycerophosphate.* The number of substances affected by ascorbic acid and H_2O_2 suggests action on a common linkage. The resemblance of the degradation of mucin to the reduction of the viscosity of starch paste by an amylophosphatase [Waldschmidt-Leitz & Mayer, 1935], the breakdown of synovial fluid mucin by serum phosphatase [Robertson *et al.* 1940] and the dephosphorylating activity of ascorbic acid [Thannhauser *et al.* 1938] suggest that a dephosphorylation may be responsible for the breakdown of mucins and polysaccharides reported above. This hypothesis is supported by the

Table 3

Reactants	mg. P liberated
1. 50 mg. β-glycerophosphate + 10 mg. ascorbic acid	0·000
2. 50 mg. β-glycerophosphate + 30 mg. H_2O_2	0·025
3. 50 mg. β-glycerophosphate + 10 mg. ascorbic acid + 30 mg. H_2O_2	0·525
4. 10 mg. ascorbic acid + 30 mg. H_2O_2	0·000

The reactants were dissolved in boiled sterile acetate buffer *p*H 5·5 and kept under oil. The total volume was 15 ml., the temperature 37° and the reaction time 24 hr.

[1] We are indebted to Dr Helen Arnold for bacteriological assistance.

observation that the dephosphorylation of β-glycerophosphate by ascorbic acid under anaerobic conditions requires H_2O_2. These experiments are presented in Table 3.

Although various types of substance are acted upon by ascorbic acid and H_2O_2, several compounds whose solutions are viscous (ovomucin, agar-agar and gelatin) were not affected by this system.

Discussion

The data presented show that ascorbic acid causes degradation of mucins only in the reduced form and in the presence of H_2O_2. However, it is impossible to conclude from the results whether ascorbic acid and H_2O_2 act separately and successively on the substrate or whether H_2O_2 is a sufficiently mild oxidant to form an active intermediary oxidation product such as that postulated by Bezssonoff & Woloszyn [1938] or by Dekker & Dickinson [1940].

Ascorbic acid has been considered a possible functional entity in the oxidation-reduction system of cells because of its strong reducing properties, yet no experiments have indicated its role either as an enzyme or coenzyme in such a system. The degradation of mucins and polysaccharides, as described in this paper, should be considered as a possible function of vitamin C in physiological and pathological processes.

Summary

1. Ascorbic acid and H_2O_2 react to cause degradation of synovial fluid mucin. This degradation involves a breakdown of the macromolecules without the liberation of detectable amounts of reducing substances or amino-sugars.

2. This action is not limited to mucins of mesothelial origin as is that of mucinase. The ascorbic acid-H_2O_2 system acts on gastric and salivary mucins, and on polysaccharides, such as starch, pectin, flaxseed mucilage and the polysaccharides of synovial mucin and cartilage, and destroys the capsules of various types of pneumococci. However, it causes no change in ovomucin, agar-agar or gelatin.

3. Ascorbic acid and H_2O_2 cause a dephosphorylation of β-glycerophosphate.

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100. THE FERMENTATION PROCESS IN TEA MANUFACTURE

7. THE INFLUENCE OF EXTERNAL FACTORS ON FERMENTATION RATE

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During the course of investigations on the mechanism of tea fermentation carried out in this laboratory in the past four years many incidental observations have been made on the effect of various factors on the rate and extent of fermentation. In this communication are summarized such of these observations as appear to have some general scientific appeal.

Factors affecting fermentation may conveniently be divided into two classes, those modifying the nature of the leaf plucked for tea manufacture, and those in which variations in the course of manufacture affect the nature of the finished product. These two classes will be considered separately.

Factors affecting the nature of the green leaf

A full study of such factors would involve variations due to season and climate, to forms of manuring, to the presence or absence of shade, and to different techniques of plucking and pruning the bushes, to mention only some of the possibilities. No one of these factors has been studied exhaustively but it is possible to state that variations in oxidase activity over the whole season show small but erratic differences, and that the effect of manuring, if it exists, is but a small one. Two other factors, coarseness of plucking and variations associated with individual bushes, have received some attention.

Coarseness of plucking. Although shoots plucked for manufacture generally approximate to 'two leaves and a bud', coarser forms of plucking are sometimes found. In the coarse forms of plucking there may be shoots consisting of three or four leaves and a bud. A shoot consisting of four leaves and a bud is illustrated in Fig. 1.

Fig. 1. Tea shoot, four leaves and a bud.

One of the most convenient methods of studying the effect of coarseness of plucking on fermentation is to determine the gradient of tannin and oxidase, that is to say, the distribution of these substances in the component parts of the shoot. A large number of shoots are divided into bud, 1st leaf, 2nd leaf etc., and

stalk. Oxidase activities of such samples are determined manometrically and expressed as initial rates of uptake per hr. for 1 mg. dry weight (Q_{O_2}), and tannin contents are estimated by the Lowenthal method.

Table 1

	Tannin % dry weight	Q_{O_2}	Moisture
Bud	26·5	20·1	75·5
1st leaf	25·9	17·5	73·0
2nd leaf	21·9	17·7	73·8
3rd leaf	18·6	17·5	73·8
Stalk	14·4	31·2	85·1

Table 1 records typical results found for these gradients. The coarser the plucking the greater is the percentage of 3rd (and 4th) leaf and stalk in the shoots and this results in a net reduction in the percentage of tannin in the shoots but the oxidase activity remains relatively unaffected. One would expect therefore that with increasing coarseness of plucking the rate of fermentation would be unchanged, but that with less tannin to be oxidized, the process would require less time.

Confirmatory experiments on shoots showed that the tannin content of the whole shoot decreased appreciably with increasing coarseness of plucking whereas oxidase activity increased only very slightly, although significantly.

The extent of tannin oxidation at a given stage in fermentation may be determined indirectly. The partly fermented tissue is ground to a paste, suspended in water, and the uptake of O_2 determined manometrically (residual uptake). If the amount of O_2 consumed in the fermentation of the fresh or withered leaf has been previously determined, subtraction of the residual uptake from the figure for fresh leaf gives the O_2 consumption, and hence proportionately the extent of tannin oxidation, in the partly fermented leaf. In this way it was shown that the percentage oxidations in two samples of leaf, differing only in their coarseness of plucking, were almost identical (64 %) after 90 min. fermentation. At no time have significant differences been observed between the times required for fermentation of finely and coarsely plucked leaf.

This is contrary to expectation and it is evident that some other factor is involved in addition to enzymic activity and tannin content. It is suggested that a permeability factor is concerned, and that on account of lower permeability in the coarser tissue, fermentation is slowed down. The possibility that permeability factors play some part in determining the rate of tea fermentation is also indicated by the variation of this rate with different degrees of withering. This will be discussed in more detail in a later section of this paper.

Varietal effects. In a previous communication [Roberts, 1939, 2] the variation of fermentation rate with jat, or commercial brand, was discussed. Significant differences between some jats were found, and this work has since been abundantly confirmed. The work of the Botanical Department at Tocklai, however, has led to the conclusion that the species, *Camellia Thea* Link, is to be considered as a linear series of forms between two extreme types, with possibilities of hybrid formation between the extremes. As one jat may contain several of such forms it is clear that the jat is not a satisfactory means of classification for scientific purposes.

It is as yet too early to state whether taxonomic intervals in such a series have any effect on oxidase activity in the tea leaf but experiments will shortly be started to investigate this possibility. Meanwhile it has been established that oxidase activity within a jat varies much more than between two jats.

Thus three jats were compared over a whole season and the average Q_{O_2} figures obtained were as follows:

Betjan	China	Kharikatia
20·8 ± 0·5	20·8 ± 0·6	17·6 ± 0·6

The Kharikatia has a significantly lower oxidase content than the other jats.

Results for leaf from single bushes within the Betjan jat show that the Q_{O_2} may vary from 18·6 to 31·4, a much greater range than the difference between fast- and slow-fermenting jats. With such heterogeneity of material within a single jat it is clear that the establishment of differences, such as are found between Kharikatia and Betjan for example, is dependent upon accurate sampling. While it is considered that sufficient attention was paid to sampling in the experiments in which fermentation rates of different jats were compared, it is now emphasized that these differences represent a comparison of two heterogeneous populations, and have no varietal significance.

Relation between Q_{O_2} of fresh leaf and its rate of fermentation. So far in these studies it has been more or less assumed that the Q_{O_2} of fresh leaf as measured manometrically gives a reasonably accurate estimation of its oxidase activity. In a series of empirical experiments, designed to measure the effects of variations of time and temperature of fermentation on the quality of different commercial varieties of tea, measurements were incidentally made of the Q_{O_2} of the fresh

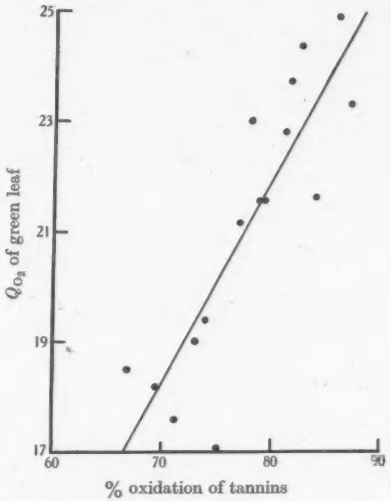

Fig. 2.

leaf and of the extent of oxidation after $2\frac{1}{2}$ hr. fermentation at 80° F. A very highly significant correlation ($r = 0{\cdot}8663$, $P < 0{\cdot}1\,\%$) was obtained between these two variables. It is evident that despite errors introduced by the impossibility of obtaining the same degree of wither in each experiment the Q_{O_2} value largely determined the rate of fermentation of the leaf under factory conditions (Fig. 2).

Variations in the course of manufacture

Withering. In the manufacture of tea in N.E. India the leaf is generally subjected to a preliminary withering process before mechanical rolling. The leaf is spread thinly, about 1 lb. per square yard, on racks and allowed to wither for about 18 hr. This process is generally uncontrolled and the extent of loss of moisture is determined by the temperature and humidity of the atmosphere and the exposure of the leaf to the wind. A normal good wither results in the reduction of the moisture content of the leaf from about 77 to 65–70 %. The leaf has then lost its turgidity and can be twisted without smashing the tissue. In addition to this physical preparation of the leaf for rolling, chemical changes occur and there are also modifications in the activities of both hydrolytic and oxidizing enzymes [Kursanov, 1935].

When oxidase activity is measured by the manometric method it is observed that withering is always associated with a loss in oxidase activity, and this loss increases progressively with the degree of wither. In one experiment the wither was controlled by varying the thickness of spread of the leaf, and in this way three different withers were obtained. Table 2 shows the variation in Q_{O_2} as a result of the different withers.

Table 2

Sample	Moisture content	Q_{O_2}
Fresh	79·10	18·4
Withered	65·35	16·6
Withered	61·50	14·2
Withered	55·60	13·4

Each determination of Q_{O_2} is a mean of triplicates in good agreement.

In all, 16 experiments were carried out in which the oxidase content of fresh leaf was compared with that of withered leaf and in each case there was a significant decrease in Q_{O_2} as a result of withering. The extent of this decrease is irregular and is probably determined to some extent by the nature of the leaf and the rate of moisture loss.

As a result of the correlation found between Q_{O_2} values for fresh leaf, and the rate of fermentation under factory conditions, withered leaf would be expected to ferment more slowly than unwithered leaf. This is not realized except in the cases where the leaf is definitely over-withered (i.e. to 50 % moisture).

Previous experiments [Roberts, 1939, 1] had shown that withering has no detectable effect on the rate of oxidation of tannins, but these results must be qualified as a result of the development of the above method for estimating the extent of tannin oxidation in partly fermented leaf by measurement of residual O_2 uptake, which gives much more accurate results than the Lowenthal titrimetric method. It now appears that withering at first slightly increases the rate of fermentation but that as the degree of wither becomes higher the rate of fermentation again falls, and that finally with very high withers (i.e. to 50 % moisture or less) the rate is very appreciably diminished.

Two series of experiments have formed the basis of these conclusions.

In the first of these a normal wither, i.e. to about 70 % moisture is compared with a very decided under-wither. The latter was realized by thick spreading of the leaf, 3 lb. to the square yard, during the whole process. Table 3 gives the residual uptakes recorded, after 2 hr. fermentation, for the two types of wither.

Table 3

Date	μl. O_2 per mg. dry weight of tissue: Normal wither	Under-wither
14. vi. 39	4·00	4·95
	4·19	4·99
	3·82	4·81
	4·46	5·08
4. vii. 39	2·74	3·50
	3·08	3·61
	3·88	4·28
	3·05	4·06
21. vii. 39	3·72	3·84
	3·33	3·94
	3·64	3·89
	Mean 3·63	4·27

Sig. diff. (P=0·1 %) 0·33

Subjecting these figures to an analysis of variance it may be shown that the residual uptake of the under-withered leaf is significantly greater than that of the normally withered leaf, on the 0·1 % level of probability. The higher residual uptake of the under-withered leaf implies that more fermentation has taken place in the normally withered leaf. The total uptake for withered leaf after fine mincing averages about 12·0 μl. O_2 per mg. dry wt. so that the uptakes at the time of sampling were 8·37 and 7·73 μl. O_2 respectively. The rate of fermentation was therefore about 7–8 % faster in the higher-withered samples. A significant difference of this order would not easily be found by the Lowenthal method with this number of repeats.

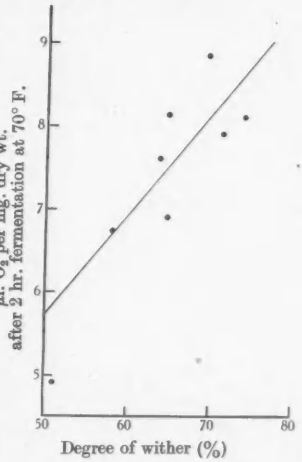

Fig. 3.

The second experiment was not primarily designed to show the relation between withering and rate of fermentation. However, in a series of manufacturing experiments carried out during 1940, data were incidentally accumulated on the extent of oxidation after 2 hr. fermentation, and the degree of wither, this latter being measured as the weight of the withered leaf as a percentage of the fresh. The results are recorded in Fig. 3 which shows a significant correlation between these two variables (r=0·7885, P=2 %). Although this correlation rests on eight observations only, it has frequently been observed during the recent manufacturing season that with over-withered leaf, i.e. leaf in which the moisture has fallen to 55 % moisture or less, the extent of fermentation in a given time is distinctly less than obtained after a normal wither. In all cases where the wither was over-high the colour of the aqueous extract of the fermented leaf was much paler than normally observed for the same time and temperature of fermentation. This indicates a lower extent of fermentation.

These experiments show therefore that the effect of withering on fermentation is not a simple one. It is suggested that as oxidase activity decreases progressively

with increasing degree of wither some permeability factor may be involved. In the early stages of withering increased permeability may reverse the effect due to decreased oxidase activity but as oxidase inactivation increases such factors can no longer compensate for this and rate of fermentation commences to fall.

I am intentionally vague about the nature of this permeability factor, but experiments indicate that it may be associated with an increase in the solubility in water of the oxidase and hence with its diffusibility within the cell.

Although the oxidase activity of tea leaf is largely associated with insoluble matter the juice expressed from green leaf contains quite a considerable amount of the oxidase. As no high-speed centrifuge is available in this laboratory it is impossible to state whether the oxidase in the juice is in true solution or in a high state of dispersion. In the following discussion therefore water-soluble oxidase will include any of the enzyme too highly dispersed to be separated out at 2000–3000 r.p.m. in the centrifuge.

During withering there is a considerable loss of moisture so that there is a concentration of solutes in the juice expressed from the leaf. The juice from withered leaf would therefore be expected to have a higher oxidase activity per ml. than juice from fresh green leaf. Measurements of the rate of O_2 uptake by juice show that the increase is distinctly greater than can be accounted for by concentration of solutes only. Results of two such experiments are quoted in Table 4.

Table 4

Leaf	Solid matter mg. per ml. juice	Initial rate of O_2 uptake μl. per hr.	
		Per mg. dry weight	Per ml. juice
Exp. 1. Fresh	88	2·5	220
Withered	164	5·3	868
Exp. 2. Fresh	71	8·8	624
Withered	141	14·8	2088

In Exp. 1 uptakes are measured for 3 ml. juice without dilution. In Exp. 2 0·5 ml. juice, diluted to 3 ml. with water, is measured into each Warburg vessel.

The faster rates of uptake in Exp. 2 are due to the concentration effect referred to in a previous paper in this series [Roberts, 1940, 2]. As the enzyme concentration in the juice from withered leaf is greater than that from fresh leaf the recorded activities in the former case are probably too low, but even without making corrections for this effect it is clear from the above figures that the water-soluble oxidase has increased considerably as a result of withering, and is at least double that to be expected if concentration of the juice by evaporation were the only effect of withering. Where the concentration of the juice, in mg. solid matter per ml., has been doubled by withering, the increase in the enzymic activity is a fourfold one. It is evident that the withering process is associated with increased dispersion of the oxidase.

Confirmation of this is found in the recently published work of Lamb & Sreerangachar [1940] who also observe an increase in the water-solubility of the tea oxidase from Ceylon leaf as a result of withering.

It is possible that the increased solubility of the enzyme, by favouring its greater dispersion, results in a slightly faster rate of tannin oxidation in the fermenting tea leaf despite a net reduction in enzymic activity. Whether this is the true explanation of the 'permeability' effect remains uncertain but until more definite information is available it will serve as a hypothesis to account for the observed facts.

Lately, in attempting to separate chloroplast material from green tea leaf by the method of Neish [1939], it has been noticed that the fractions obtained have a very considerable oxidase activity. Unfortunately these fractions were not of purely chloroplastic origin but it is tempting to conclude that oxidase activity within the tea leaf may be associated with chloroplastic matter. Now it has been shown by Bokuchava [1935] that there is a marked fall in the chlorophyll content of the tea leaf as a result of withering. There is thus some evidence suggesting that the net fall in oxidase activity as a result of withering, and possibly the increased dispersion of the enzyme, are associated with degradation of the chloroplasts.

Temperature. The rate of tannin oxidation in suspensions of finely ground tea leaf tissue was found by Harrison & Roberts [1939] to be increased by only some 30 % when the temperature was raised from 25° to 40°. The probable effect of temperature differences on the degree of condensation of the tannins in fermented tea was also mentioned.

During the manufacturing seasons of 1939 and 1940 these effects have received considerable attention and the original statements can now be somewhat amplified. Discussion of the effect of temperature difference on condensation of tannins will be reserved for a communication now in course of preparation.

It must be emphasized that the results of manometric experiments on the variability of fermentation rate with temperature do not necessarily apply to factory conditions, as substrate concentrations and degree of condensation of tannins are both affected by the suspension of the tissue in excess of water. Failing direct methods the residual uptake method has been employed to study the effect of temperature on fermentation. Measurements of the extent of fermentation are made after a period of 2 hr. The employment of a difference method, such as the determination of the residual uptake, affects the accuracy of these measurements and there are further obstacles to the accurate determination of the extent of oxidation in the absence of strict control of temperatures in the earlier stages of manufacture.

In the miniature factory at Tocklai the withered leaf is mechanically rolled for 30 min. and further bruising is then caused by passing through a McKercher C.T.C. machine. The leaf is then bulked and sampled before transferring to air-conditioned cabinets for the fermentation at controlled temperatures. Reference to Tables 5 and 6 will show that after a fermentation time of 2–2½ hr., which includes the preliminary rolling and subsequent treatment, considerably more than half the total oxidation has taken place. This period of fermentation is overlong to compare rates with accuracy, but shorter periods cannot be used owing to the absence of proper control over the temperature for the 1st hr.

As yet we have found no rapid method of bringing about the necessary damage to the shoots. A coarse mince is not completely effective as a large proportion of the leaf escapes damage as is shown by the persistence of anaerobic fermentation [cf. Deb & Roberts, 1940]. As a means of estimating the variability of enzymic activity at different temperatures the author therefore makes no claim of high precision for this method. However for practical purposes in the factory it does measure the effect of different temperatures of fermentation with some accuracy, and as such is of considerable value.

The results obtained by this method will now be discussed and it will be seen that, despite its imperfections, conclusions of some importance can be drawn.

In the 1939 experiment 16 comparisons were made between the extent of oxidation after 2½ hr. fermentation at 80° and 90° F. respectively. These results are recorded in Table 5.

Table 5

μl. O_2 per mg. dry weight.

Total uptake of fresh leaf	Residual uptakes after 2½ hr. fermentation		Uptakes up to the time of sampling	
	80° F.	90° F.	80° F.	90° F.
11·64	2·24	1·98	9·40	9·66
11·30	1·93	1·70	9·37	9·60
11·92	1·59	1·22	10·33	10·70
12·67	2·75	1·40	9·92	11·27
11·09	2·00	1·86	9·09	9·23
12·55	2·69	2·58	9·86	9·97
11·81	2·76	2·62	9·05	9·19
12·59	3·27	2·94	9·32	9·65
11·58	1·52	1·81	10·06	9·77
12·23	2·54	2·48	9·69	9·75
11·60	3·55	3·05	8·05	8·55
11·75	3·95	3·76	7·80	7·99
11·95	1·89	1·93	10·06	10·02
12·09	3·03	3·34	9·06	8·75
12·82	3·48	3·17	9·34	9·65
12·72	3·70	3·78	9·02	8·94
			Mean 9·34	9·54

The increase in oxidation at the higher temperature of fermentation just falls short of significance. The value for 't' is 2·115 whereas 't' should equal 2·131 for significance on the 5% level of probability.

In the 1940 experiments two sets of comparisons were made at 60° F. compared with 80° F., and 70° F. with 90° F. Each treatment was replicated three times on three different occasions. It was impossible to compare the four temperatures of fermentation on the same day so that each pair of comparisons must be considered separately. Table 6 records the uptakes, obtained by difference as in Table 5, after 2 hr. fermentation at each temperature.

Table 6

μl. O_2 per mg. dry weight.

Date	60° F.	80° F.	Date	70° F.	90° F.
20. viii. 40	6·70	8·04	13. viii. 40	8·30	8·77
	7·35	8·20		8·01	8·89
	7·81	8·10		8·01	9·01
10. ix. 40	5·80	7·53	3. ix. 40	7·59	8·82
	7·08	7·58		8·14	8·32
	5·62	7·75		7·98	8·28
24. ix. 40	6·70	9·06	17. ix. 40	6·68	6·88
	7·46	9·51		6·93	7·38
	7·08	9·47		6·68	7·51
Mean	6·84	8·36		7·59	8·21
Sig. diff. $P=5\%$	0·45			0·25	

The conclusions we can draw from these results are as follows:

(1) There is a marked increase in the rate of fermentation when the temperature is increased from 60° to 80° F.

(2) The acceleration in the rate for the 20° F. rise between 70° and 90° F., although significant, is distinctly less than between 60° and 80° F.

(3) An increase in fermentation rate above 80° F. is small and also just short of statistical significance.

The data are insufficient to construct an enzymic activity-temperature curve but it is clear that the maximum temperature for the enzyme system is not far removed from 85° F. This low maximum temperature accounts for the abnormally small increase in enzymic activity between 25° and 40° previously established for suspensions of ground tea leaf tissue.

It is rather unusual to find so low a maximum temperature as this for an enzyme. Experiments were therefore undertaken to determine the extent of enzymic inactivation during the course of fermentation.

The method employed for estimating oxidase activity of partly fermented leaf consists of adding 3 ml. of a green leaf infusion (50 g./500 ml.) to 150 mg. of the finely ground fermented leaf and recording O_2 uptakes over a period of 30 min. As unoxidized substrate is present in excess the uptake should be proportional to oxidase activity.

Results for three different times and two temperatures of fermentation are given in Table 7. All results are means of triplicates in good agreement.

Table 7

Uptakes, μl. O_2/30 min. per mg. dry weight.

hr. ...	2	$4\frac{1}{4}$	$5\frac{3}{4}$
70° F.	9·5	8·9	8·9
90° F.	8·4	6·6	5·1

Very little enzymic inactivation is apparent at 70° F., the figures being very little lower than those recorded for withered unfermented leaf. At 90° F., however, enzymic inactivation is quite marked and after $5\frac{3}{4}$ hr., about half the original activity only is retained. It is suggested that the higher condensation products of oxidized tea tannin are responsible for the inactivation of the enzymes. At 70° F. even after $5\frac{3}{4}$ hr. fermentation the degree of condensation is not high and the oxidase remains, comparatively speaking, uninhibited.

Although marked inactivation of oxidase occurs at 90° F. this is not sufficient after 2 hr. fermentation to account for the relatively low temperature coefficient between 80° and 90° F. It therefore appears that the oxidase of tea leaf has in fact an abnormally low maximum temperature, and that inactivation of the enzyme by the products of its activity is only of secondary importance.

The maximum temperature of the tea oxidase has also been studied by Lamb & Sreerangachar [1940], and it was shown that the water-insoluble form of the oxidase had a maximum temperature of 27° in excellent agreement with my figure for the whole fermentation process. The conditions under which the Ceylon experiments were carried out were rather different from those in my experiments, for the rate of tannin oxidation was determined in relatively dilute solution and the leaf was obtained from bushes living under much more temperate conditions. Although it is not certain that these differences would materially affect the maximum temperature of an enzyme it is possible that the agreement found may later prove to be less complete.

Fermentation in O_2. Results have already been published [Roberts, 1940, 1] which show that tea fermentation as measured manometrically is accelerated in an atmosphere of O_2. Factory experiments confirm this acceleration. If mechanically rolled leaf is placed in a drum through which O_2 is passing, oxidation of the tannins is brought to the same stage in 25 min. as in a period of 2 hr. or more in a normal atmosphere (Table 8).

The extents of oxidation in the two processes are comparable, amounting to 77 and 75 % completion in the control and oxy-fermentation processes respectively.

Table 8. *Residual O_2 uptakes of normally fermented teas and teas fermented for 25 min. in O_2*

μl. O_2 per mg. dry weight.

Date	Control	Fermented in O_2
24. v. 40	3·60	4·40
28. v. 40	3·10	3·00
29. v. 40	2·53	3·23
19. vi. 40	2·51	2·23
23. vii. 40	2·51	3·03
24. vii. 40	2·48	2·48
15. xi. 40	2·73	2·97
15. xi. 40	2·07	2·17
Mean	2·69	2·94
Uptakes during fermentation	9·31	9·06

Bacterial contamination. The bacterial flora normally found associated with the tea leaf have no significant effect on the rate or extent of fermentation although Benton [1938] has recorded the adverse effect on quality resulting from heavy contamination and has observed a sharp drop in bacterial numbers on crushing the leaf. This the author attributes to the bactericidal effects of *o*-quinones formed in the course of fermentation. The concentration of *o*-quinone, as a short-lived intermediate product, will be proportional to its rate of production and hence to the rate of O_2 uptake. In agreement with this it is found by Benton that bacterial numbers tend to rise again in the closing stages of fermentation, i.e. when *o*-quinone concentration is again low.

If the bacterial population is brought to levels considerably greater than would normally be encountered in the course of manufacture, the rate of tannin oxidation is accelerated, as is indicated by a significant decrease in the residual O_2 uptake of partly fermented samples. In one experiment the residual O_2 uptake of the sample with the high bacterial population was $2{\cdot}0 \pm 0{\cdot}23$ μl. per mg. dry wt. after 2 hr. fermentation, whereas the residual O_2 uptake of the control sample was $3{\cdot}0 \pm 0{\cdot}17$ μl. This difference indicates that oxidation of tannins in the artificially infected sample is distinctly more rapid. The direct manometric method shows no effect of bacteria on fermentation rate. It is thought that the effect may be due to local development of NH_3 leading to autoxidation of the tannins. If the tissue is suspended in excess water such effects cannot be realized as any NH_3 formed would be immediately diluted and rendered non-effective.

Discussion and summary

A consideration of all the above results leads to the conclusion that the oxidase activity, as determined by the direct manometric method, does not always completely determine the rate of tea fermentation. Other factors may also play some part and the only accurate method of measuring fermentation rates is that referred to in this communication as the residual uptake method. The reliability of this method is shown by the fact that when the later stages of fermentation are carried out in the Warburg apparatus the rate and extent of oxidation is the same no matter how the initial stages were carried out. Thus if factory-treated leaf is fermented until half of the tannins are oxidized, and then ground to a paste and suspended in water in Warburg vessels, the O_2 uptakes then recorded are precisely the same as those found for the latter half of the fermentation when the process is carried out entirely in the Warburg apparatus.

The residual uptake method enables us to determine how far the oxidase content controls the rate of fermentation.

The high correlation ($r = 0{\cdot}8663$) found between the Q_{O_2} values for fresh green leaf and the rate of fermentation of this leaf under factory conditions shows that it is the oxidase activity which largely determines the rate of fermentation. In these experiments the wither was a variable and uncontrolled factor, but in no case was the leaf withered to less than 65% moisture so that the effect due to withering was a comparatively small one. The differences in fermentation rates between different jats (Betjan and Kharikatia) so frequently referred to in this series is another case where the oxidase activity is the most important factor controlling the process. The increase in rate of fermentation in O_2 is also approximately proportional to the increase of Q_{O_2} of the fermenting leaf as determined manometrically.

These however are the only cases where a direct measurement of Q_{O_2} affords an accurate estimate of the speed of the fermentation process under factory conditions.

In the other cases investigated, other factors in addition to oxidase activity are affected and rate of fermentation is no longer so closely correlated with the Q_{O_2} of the leaf, as determined manometrically. Permeability factors may vary as in the case of shoots varying in their coarseness of plucking. A loss in total oxidase activity during withering may be partly offset by a greater ease of diffusion of the enzymes within the tissue. Where rates of fermentation vary with temperature entirely different results may be obtained by the direct manometric and the residual uptake methods. At 36° little oxidase activity is lost by the time tannin oxidation is complete when the fermentation is carried out in Warburg vessels containing 200 mg. of tissue suspended in 3 ml. water. On the other hand at 32° (90° F.) the results show that roughly half of the oxidase is destroyed when the fermentation takes place under factory conditions. It appears that, as a result of the dilution with water, the tannins are less readily condensed in the former case and hence enzymic inactivation is less marked. The increase in oxidase activity with temperature is therefore likely to be less marked under factory conditions than would be expected from the results of manometric experiments.

A third way in which fermentation rates may be affected without apparently affecting the Q_{O_2} values is shown in the experiment with bacteria. The significant increase in the rate of tannin oxidation that occurs after a heavy inoculation with bacteria is not observed when the tissue is suspended in water.

The author wishes to express his thanks to the Director, Mr P. H. Carpenter for the encouragement he has received during the course of this work and to the Indian Tea Association for permission to publish these results. Thanks are also due to Dr Wight for permission to quote from his hitherto unpublished work in connexion with the part of this paper dealing with varietal effects.

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101. A DIETARY FACTOR CONCERNED IN COPROSTEROL FORMATION

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In spite of the progress made in recent years in the chemistry of cholesterol, little is known about its metabolism, so that even the mechanism of its biological conversion into coprosterol remains still unexplained. The excretory origin of coprosterol, taken in conjunction with the fact that it differs from cholesterol in elementary composition merely by two additional hydrogen atoms, obviously led the early investigators to the assumption that hydrogenation of cholesterol was effected by intestinal bacterial action.[1] There is indeed convincing experimental evidence that coprosterol originates in the large intestine, but there is equally conclusive proof that, even under the most favourable conditions, intestinal bacteria are unable to hydrogenate cholesterol *in vitro* (for recent unsuccessful attempts see Schoenheimer *et al.* [1930] and Dam [1934]). Moreover, direct hydrogenation to coprosterol, a *cis*-decalin derivative, is inherently improbable, since under all known conditions of hydrogenation cholesterol yields only the isomeric dihydrocholesterol (β-cholestanol), a *trans*-decalin derivative.

We suggested some time ago [Rosenheim & Starling, 1933] that coprosterol may arise in the intestine not as a product of direct hydrogenation of cholesterol itself, but through that of an intermediary oxidation product, the ketone cholestenone. The primary oxidation product of cholesterol, the α-glycol *cis*-3:4-Δ^5-cholestenediol, easily furnishes cholestenone by loss of water, the reaction involving a shift of the ethylenic linkage from C_5 to C_4 and thereby enabling a transition to the *cis*-decalin derivative coprosterol on hydrogenation. In feeding experiments with these oxidation products we found that they were excreted as coprosterol [Rosenheim & Webster, 1935; Rosenheim & Starling, 1937]. Similar results were obtained by Schoenheimer *et al.* [1935] and by Anchel & Schoenheimer [1938], who made use of the ingenious device of 'labelling' cholestenone with deuterium.

We have since attacked the problem from another side by investigating a hitherto neglected incidental observation, made over 30 years ago, which suggests a relationship between diet and coprosterol formation. In their study of cholesterol metabolism Dorée & Gardner [1908] kept a dog on various mixed diets of meat and vegetables. Cholesterol only was excreted by the dog under these conditions. On account of its large cholesterol content brain seemed to be a convenient medium for the administration of cholesterol, and Dorée & Gardner found, in a single experiment with raw brain as the exclusive diet, that the faecal sterols consisted mainly of coprosterol. No explanation for this remarkable result was available at the time and the experiment was not repeated.

[1] For the sake of historical accuracy it should be noted that the excretory origin of coprosterol was first recognized by W. Marcet, F.R.S. [1857; 1860], a physician at Westminster Hospital. The method used by Marcet for its isolation was essentially the same as that employed by later workers, and the melting point, 96°, of his 'excretin' is identical with that given for coprosterol by Bondzinsky [1896], who is usually credited with the discovery.

In a preliminary experiment we kept two dogs on a diet of raw brain, but had to discontinue the experiment as the animals suffered severely from diarrhoea, in spite of a liberal supply of bones as roughage. A second attempt with two dogs of a different breed had the same result, the dogs rapidly losing weight. When we used rats, however, as experimental animals and replaced raw brain by cooked brain mixed with bone meal, they excreted large quantities of coprosterol. As much as 40 g. of fairly pure coprosterol, M.P. 95–96°, were obtained in 4 days from 100 rats. Possibly the feeding of cooked brain to dogs would give similar results, but for various reasons we continued the work with rats on a synthetic diet.

On the sterol-free standard diet the rats excreted only traces of coprosterol. When cholesterol to the extent of 1–2 % of the diet, dissolved in lard and mixed with the diet, was given, it was excreted unchanged. On replacing part of the cholesterol by cooked brain, however, large amounts of coprosterol appeared in the faeces. It became thus evident that brain contains a substance or factor which conditions the conversion of cholesterol taken by the mouth into faecal coprosterol. A cholesterol-free brain powder was subsequently prepared by exhaustively extracting minced brain of ox or sheep with cold acetone. On giving this powder, mixed with the diet containing 1–1·5 % cholesterol, the rats excreted coprosterol to the extent of 70–90 % of the total faecal sterols. Systematic experiments then showed that the factor is thermostable, insoluble in water, acetone, ether or petrol, but soluble in hot 85 % alcohol. It was present in the 'white matter' of Thudichum, which is deposited when hot alcoholic brain extracts are cooled, and which consists of the cerebrosides phrenosin, kerasin and nervon, the phosphatide sphingomyelin etc.

The disentanglement of the individual constituents of 'white matter' is laborious and their preparation in a pure condition and in sufficient amounts for feeding experiments requires larger supplies of 'white matter' than we had available. Although our preparations of phrenosin, the most abundant of the cerebrosides, were not analytically pure, they showed a high degree of activity. Fortunately we had at our disposal a specimen of pure phrenosin, prepared more than 60 years ago by its discoverer Thudichum [see Rosenheim, 1930]. When Thudichum's phrenosin, in amounts proportional to that contained in the brain powder, was added to the standard diet plus cholesterol, the rats excreted coprosterol to the extent of over 80 % of the faecal sterols. In a single experiment Thudichum's kerasin was inactive, as also were lecithin, kephalin and choline.

A preliminary survey of the distribution of the factor showed that brain was the richest of all the organs examined and that pancreas, kidney and liver contained it in much smaller amounts and in decreasing order. It is present in egg yolk, but absent from muscle (horse flesh) and from milk. The distribution of the factor thus seems to run parallel with the cerebroside content of the various organs and foods examined.

Experimental

Rats of the Institute strain of Wistar rats and of the average weight of 200 g. were used. Groups of 3 to 6 rats, with the same number as controls, served for each experiment. The animals were housed in cages with coarse wire-grid bottoms which allowed the faeces to drop on to blotting paper below. The rats were kept on the diet for a few days before the start of the experimental period, which usually lasted for 7 days. The faeces were collected during the experimental period only, no quantitative sterol balances being attempted.

The standard diet consisted of

Casein (Glaxo C)	20 parts	+20 mg. cod liver oil per rat, per day
Rice starch	72 ,,	
Yeast powder (B.D.H.)	6 ,,	
Salt mixture	2 ,,	

supplemented with 15 g. of lard per 100 g. of the diet. Cholesterol, 1–1·5 % of the diet, was dissolved in the lard and incorporated with the diet. The average food consumption per rat, per day was 10–12 g.

For analysis the faeces, collected daily and preserved in 40 % KOH, were worked up by Fex's [1920] method and saponification was completed by treating the ether extracts with sodium ethoxide for 24 hr. Total sterols were determined gravimetrically by the digitonin method and saturated sterols by Schoenheimer's [1930] method. Coprosterol was recovered from the saturated digitonides by the pyridine-ether method.

Coprosterol excretion on brain diet. A series of experiments was carried out in which rats were kept exclusively on a diet of cooked sheep's brain. The brain was steamed for ½ hr. in a steam cooker, the supernatant fluid decanted and the remaining mass thoroughly mixed with bone meal (4 g. to 100 g. of fresh brain). The diet was prepared daily and readily taken by the animals, 10–15 g. being consumed per rat, per day. The brain diet, given for relatively short periods, did not produce any signs of ill health, but the animals lost slightly in weight.

In a typical experiment 12 rats received 200 g. of brain per day for 7 days and excreted 20·5 g. of sterols, of which 17·0 g. (82·9 %) were saturated sterols. The unsaponifiable portion of the ethereal extract was fractionally recrystallized from methyl alcohol and yielded 12·5 g. colourless needles of coprosterol, M.P. 100–101°, $[\alpha]_D^{20°}$ +23·5°. A mixed melting point with authentic coprosterol, prepared by catalytic reduction of cholestenone [Ruzicka *et al.* 1934], showed no depression. The benzoate, obtained by treatment of coprosterol with benzoyl chloride in pyridine, had M.P. 124–125°, and the acetate, M.P. 89–90°. A small quantity (0·4 g.) of impure cholesterol, M.P. 138–140°, was isolated from one of the fractions.

In a large scale experiment 100 rats consumed 5·5 kg. of brain in 4 days. They excreted 56·5 g. of sterols, of which 85·6 % were saturated sterols. The coprosterol excretion per rat, per day was 120 mg. The unsaponifiable fraction of the ether extract, worked up in the usual way, yielded 41·4 g. of crude coprosterol, M.P. 96–97°, and 0·5 g. of cholesterol, M.P. 146–147°.

The bulkiness of the faeces of rats on a brain diet was a constant feature in these experiments. The increase in volume and weight is partly due to undigested neurokeratin, present in brain to the extent of 15–20 %, and partly to the excretion of cleavage products of certain brain constituents (see below). Since the increased 'roughage' of the diet might have favoured coprosterol formation, control experiments were made in which cellulose (filter paper), bone meal or calcium phosphate was added to the diet together with cholesterol. The results of these experiments showed that 'roughage' had no effect on coprosterol excretion (see Table 1). An increase in the amount of 'unsaponifiable' ran parallel with the increase in weight of the faeces of rats on a brain diet. This increase was traced to the presence of a substance crystallizing in sphaerocrystals, which was isolated in considerable amounts (*ca.* 20 g.) from the ethereal extracts before or after saponification. The sphaerocrystals are readily soluble in hot methyl alcohol, acetone and ethyl acetate, and are easily separated from coprosterol owing to their insolubility in chloroform and petrol. In its melting point, 87–89°,

and properties the substance resembles lignoceryl sphingosin, a partial cleavage product of kerasin, which occurs in liver [Thannhauser & Fraenkel, 1931], lung and spleen [Tropp, 1935] and has also been found in serum in a case of hypercholesterolaemia [Schoenheimer, 1933]. In view of its possible bearing on the mechanism of coprosterol formation the substance is being further investigated.

Coprosterol excretion on the standard diet etc. The amount of sterols excreted on the standard diet was found to be so minute that a large number of rats had to be used in order to obtain reliable quantitative results. When 23 rats were kept for 7 days on the diet, they excreted 1·02 g. sterols of which 0·47 g. (46 %) consisted of saturated sterols. From the digitonide obtained after bromination in alcoholic solution, coprosterol, M.P. 96–97°, was isolated by the pyridine-ether method, the average amount per rat, per day being only 3 mg.

The effect of the addition of cholesterol to the standard diet was investigated by using a batch of 60 rats, in groups of 6 rats each, which served also as controls in other experiments. Cholesterol, 1 g. per day for each group, was incorporated in the diet. The total sterol excretion amounted to 38·3 g. of which 29·8 g., or nearly 80 %, consisted of cholesterol, which was readily isolated in typical crystals, M.P. 148–149°. Coprosterol was obtained from the saturated digitonides as above described, the average daily excretion per rat, 18 mg., being somewhat higher than on the standard diet alone.

Preparation of cholesterol-free brain powder etc. Minced ox brain (12 kg.) was treated at room temperature for 24 hr. with acetone (10 l.), filtered through linen on large Büchner funnels and the extraction repeated 5 times. After the 6th extraction and filtration the mass was freed from acetone in evacuated desiccators and finely ground in a mill. Four more extractions of the powder with 5 l. acetone each were made, the last extract yielding only traces of cholesterol. The final product, a cream-coloured powder, weighed 2 kg. Crude cholesterol (350 g.) was obtained from the acetone extracts. Cholesterol-free powders of pancreas, liver and egg yolk were prepared by the same technique.

Brain powder free from cholesterol and unsaturated phosphatides (lecithins and kephalins) was prepared by extracting minced brain (12 kg.) as above

Table 1

				Cholesterol intake		Sterol excretion				
						Total		Coprosterol		
Supplement	Per rat, per day g.	No. of rats	Days	Total g.	Per rat, per day mg.	g.	Per rat, per day mg.	g.	Per rat, per day mg.	% of total
Brain powder (ox) (cholesterol-free)	1·6	3	7	3·4	160	2·52	120	1·86	89	74
,,	1·5	3	7	3·4	160	2·51	120	2·22	105	89
Brain powder (sheep)	1·6	3	7	3·4	160	2·40	115	1·77	84	74
Brain powder (ox) (cholesterol and lecithin etc. free)	1·6	6	7	5·8	138	2·81	67	2·29	55	83
,,	1·0	3	21	9·1	145	6·48	102	5·67	90	88
Pancreas powder (cholesterol-free)	2·0	3	7	3·0	145	1·50	71	0·73	35	49
Egg-yolk powder (cholesterol-free)	2·0	3	7	3·0	145	1·71	81	0·78	37	45
Kidney (steamed)	3·5	6	7	7·0	167	4·07	95	1·35	32	33
Liver powder (cholesterol-free)	2·5	6	7	6·9	165	3·00	71	0·82	20	28
Horse flesh (steamed)	5·0	6	7	7·0	167	3·62	86	0·53	13	15
Cellulose	0·8	6	4	3·9	167	1·94	81	0·30	13	16

5 times with acetone, followed by immediate treatment with 10 l. ether at $\pm 0°$. The intermediate drying procedure was omitted in order to avoid oxidation of the unsaturated phosphatides. After 5 ether extractions, the last with 7·5 l., had been made, the mass was freed from ether and passed through a mill. The ether extracts deposited a small amount of 'white matter' on standing at $-4°$ which was mixed with the remaining brain powder. Petrol, B.P. 40–60°, was successfully used several times in place of ether, but its use was given up after it had been found on one occasion that the brain powder had been rendered inactive.

Some of the results of feeding rats on the standard diet with these powders together with cholesterol are recorded in Table 1.

The strikingly large conversion of cholesterol into coprosterol by rats on a diet containing brain powders is demonstrated by these results and contrasts strongly with the low coprosterol excretion on a meat diet (horse flesh), which is of the same order as that of the controls (see above).

Preparation of 'white matter', phrenosin etc. After the removal of cholesterol and unsaturated phosphatides, the finely ground brain powder (500 g.) was treated with 85 % alcohol (2·5 l.) for 1 hr. in a water bath at 45–50°, filtered and the extraction repeated three times. On cooling to room temperature the extracts deposited 'white matter' which was obtained as a fine white powder after filtering and drying in a desiccator. Details of feeding experiments with two of these preparations and the cerebrosides isolated from them are given in Table 2.

Phrenosin. The cerebroside fraction of the 'white matter' was separated from sphingomyelin by means of pyridine [Rosenheim, 1914]. The phrenosin fraction, still containing kerasin after fractionation from acetone at 36°, was further purified by two recrystallizations from mixtures of chloroform-acetic acid (2 : 3). The specimens of phrenosin so obtained were still contaminated with appreciable amounts of kerasin, as shown by the selenite plate test [Rosenheim, 1914], but contained only traces of sphingomyelin. Lack of material prevented further purification. The rats received daily doses of 150–300 mg. intimately mixed with the diet.

Thudichum's phrenosin. The following description of its preparation was recorded in Thudichum's own handwriting on the label of the bottle:

'From *Human Cerebrin Mixture* which had been exhausted with cold ether and then passed treatment with $PbAc.NH_3$ in spirit solution. This is *Phrenosin* which had been recrystallized from spirit only a great number of times, filtering at 28°, thus separating all Kerasin, until in the filtrate no more of the latter appeared. This portion deposited above 28° throughout and is probably free from kerasin—moist with spirit. In the last filtrate from kerasin $CdCl_2$ produced no more precipitate.'

The bottle had been so efficiently stoppered that its contents were still 'moist with spirit' on opening. The white lumps of phrenosin (60 g.) were ground up with spirit (600 ml.), warmed to 36°, kept at this temperature for 24 hr. and filtered. The alcoholic filtrate remained clear on cooling to $-4°$ and did not contain any kerasin. The white precipitate was washed with alcohol, acetone and ether and was finally obtained as a fine powder. The substance had M.P. 210–212°, $[\alpha]_D^{20°} + 4·55°$; $[\alpha]_{5461}^{20°} + 5·0°$ in pyridine ($c = 5·800$, $l = 4$ dm.). The selenite-plate test showed complete absence of kerasin. On hydrolysis by the technique described by Rosenheim [1913] phrenosinic acid, sphingosine and galactose were obtained in the usual amounts. Judged by modern standards therefore, this specimen of Thudichum's phrenosin is as pure as any prepared since by later investigators.

Thudichum's kerasin had also been obtained from human brain, no details of its preparation appearing on the label. The white product was fractionally crystallized from 10 vol. chloroform-acetic acid mixture (2 : 3). The fraction depositing below 28° had the characteristic properties of kerasin, setting to a translucent jelly from solvents on cooling, waxy when dry, but easily powdered to a white powder, M.P. 185°. In contrast to phrenosin the substance is laevorotatory in pyridine solution: $[\alpha]_D^{19°} - 3{\cdot}11°$; $[\alpha]_{5461}^{19°} - 4{\cdot}0°$ ($c = 5{\cdot}190$; $l = 4$ dm.). The selenite-plate test showed sphaerocrystals exclusively of the typical kerasin type. Only one experiment could be carried out with this preparation, which gave a decisively negative result.

Various preparations of more or less pure sphingomyelin were obtained from the 'white matter' during the pyridine fractionation process. They sufficed for only three feeding tests which gave inconclusive results. One specimen which contained a considerable amount of cerebrosides gave as much as 90% coprosterol in doses of 250 mg. per rat, per day, whilst another much purer specimen, in doses of 150 mg., proved to be inactive. Of the final cleavage products of the cerebrosides, galactose had no influence on coprosterol excretion. Only a few experiments have so far been made with the fatty acid components of phrenosin and kerasin. Phrenosinic acid, M.P. 102–103°, in doses of 150 mg. per day, yielded 58% coprosterol in one experiment, but its ethyl ester, M.P. 65–66°, was inactive in doses of 180 mg. Presumably the ester was not hydrolysed by the rat organism, as the fatty acid contents of the faeces showed an abnormally high figure in this case. The ethyl ester of lignoceric acid, M.P. 56–57°, was equally inactive. The basic component of phrenosin, sphingosine, could not be tested since the animals consistently refused to eat the diet containing either the free base or the sulphate. Choline hydrochloride had no effect on coprosterol formation in daily doses of 100 mg.

Table 2

				Cholesterol intake		Sterol excretion				
						Total		Coprosterol		
Supplement	Per rat, per day mg.	No. of rats	Days	Total g.	Per rat, per day mg.	g.	Per rat, per day mg.	g.	Per rat, per day mg.	% of total
White matter	330	3	7	3·3	157	2·01	96	1·75	84	87
,,	150	3	7	3·2	152	2·01	96	1·62	77	80
Phrenosin	300	3	7	3·1	148	1·67	79	1·38	66	83
,,	150	3	7	3·4	162	2·31	110	1·19	57	52
Thudichum's phrenosin	300	3	7	3·4	162	2·32	110	1·86	89	81
,,	330	3	7	3·4	162	2·13	101	1·75	83	82
,,	300	3	20	12·0	200	6·32	105	5·15	86	82
,,	165	3	7	3·4	162	2·21	105	1·13	54	51

The results recorded in Table 2 clearly show that the dietetic factor is soluble in hot 85% alcohol and is contained in the 'white matter' deposited on cooling the extracts. A tenfold concentration of the factor in the 'white matter' had been effected, daily doses of 150 mg. of the latter being as active as 1·5 g. of the cholesterol-free brain powder. Phrenosin is also highly active, but it is evident that it does not account for the whole activity of the 'white matter', since both Thudichum's and our own preparations of phrenosin had appreciably less effect on coprosterol excretion in daily doses of 150 mg. than the same doses of 'white matter'.

Discussion

Vague statements frequently occur in the literature regarding cholesterol metabolism, suggesting that the excretion of coprosterol is connected in some undefined manner with the kind of diet consumed. The experimental evidence now available shows that the presence in the diet of a definite chemical substance, or substances, is essential for the functioning of the mechanism concerned in coprosterol formation and a firm basis is thus provided for further investigation.

Meanwhile, existing views can be considerably clarified. Thus it becomes increasingly clear that the belief in the reducing activity of intestinal bacteria as the sole causative agent in coprosterol formation can no longer be upheld. For in numerous feeding experiments with cholesterol under conditions in which there was no reason to doubt that the usual bacterial flora was at work, we found that practically the whole of the cholesterol of the food was excreted unchanged so long as the factor was missing. It might, indeed, be suggested in explanation of this fact that a different type of bacterial flora develops under the influence of the factor. No evidence for this was obtained, however, in any of the cases in which the animals excreting coprosterol ceased to do so when the factor was withdrawn or resumed the excretion when the factor was added to the diet. Müller's [1900] early and somewhat primitive experiment demonstrating the now well-established fact that cholesterol only is excreted by adults on a milk diet, is traditionally quoted in support of the suggestion that the reduction to coprosterol is effected by certain bacteria. It would be more difficult to explain on these lines the fact that cholesterol, and not coprosterol, is the main constituent of the faecal sterols in animals receiving an exclusive diet of such a generally adequate bacterial nutrient as lean meat [see Table 1; Dorée & Gardner, 1908; Ammundsen, 1936]. Both observations find a more immediate explanation in the fact that cerebrosides are absent from both milk and muscle.

At this preliminary stage of the enquiry it would be premature to assume that the cerebrosides, and phrenosin in particular, are the only active constituents of 'white matter'. Nor is experimental evidence as yet available to supply an interpretation for the mode of action of the factor which would make its connexion with the cholestenone mechanism intelligible. The relatively large amount of active substance required to convert a given amount of cholesterol into coprosterol suggests a stoichiometric relationship between the two substances rather than a catalytic or vitamin-like activity of the factor. It may be suggested that the metabolism of the cerebrosides is interlocked with that of cholesterol esters and fats by a process of exchange-esterification of the kind envisaged by Sobotka [1938] as being concerned in the transport of fatty acids. Subsequent interaction of lipolytic and oxidative enzymes would then lead to the intermediate formation of cholestenone and its final hydrogenation to coprosterol in the intestine. This suggestion is supported by experiment in so far as we found that, in the absence of fat from the standard diet, phrenosin was unable to effect coprosterol formation and cholesterol only was excreted.

Arising out of the consideration that coprosterol excretion in man and omnivorous animals might be of the nature of a protective mechanism, enabling the organism to rid itself of the excess of cholesterol supplied in the food, it was of interest to investigate the possible inhibitory effect of phrenosin on cholesterol infiltration of the liver and the production of 'fatty livers' which result from prolonged cholesterol administration. A test of this possibility gave a completely negative answer. In a series of experiments in which rats were kept for 3 weeks on diets containing 2 % cholesterol with the addition of brain powder

in some cases and phrenosin in others, it was found that brain powder completely prevented 'fatty liver' formation, but that with phrenosin in the diet, the fat and cholesterol content of the livers was as high as that of the livers of the control animals. The brain powder was free from cholesterol and lecithins, but rich in sphingomyelin. The choline content of the latter was clearly responsible for the preventive or curative effect of the brain powder, since the same result was produced by the administration of a sphingomyelin preparation together with cholesterol.

Summary

A dietetic factor is contained in brain and other organs, but not in muscle and milk, which is essential for the conversion of cholesterol into coprosterol by the animal organism.

The factor is thermostable, insoluble in water, acetone, ether or petrol, but soluble in hot 85 % alcohol.

Thudichum's 'white matter', deposited on cooling alcoholic extracts of brain powder (free from cholesterol and unsaturated phosphatides), is rich in the factor.

A specimen of Thudichum's original phrenosin from human brain, as well as other preparations of phrenosin from ox brain, effected a conversion of cholesterol into coprosterol to the extent of 80 % of the total faecal sterols.

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102. THE METABOLISM OF β-SITOSTEROL

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THE views held on the metabolism of sitosterol have undergone considerable modification since the time when phytosterols were recognized as distinct from cholesterol, the characteristic sterol of animal organs and tissues. The earlier investigators assumed that the plant sterols, when taken up with the food by the animal organism, were converted into cholesterol, which itself was considered to be of exogenous origin. Modern opinion, as summed up in leading monographs, however, holds that all phytosterols, including sitosterol, are treated as 'ballast' by the animal organism and excreted unchanged. Without entering into a detailed discussion of the extensive literature, it may be said that much of the evidence on which this view is based is unconvincing and indirect. Moreover as far as sitosterol is concerned, the experimental work has been carried out with what are now known to have been mixtures of various sterols, such as the sitosterols of wheat or soya beans. The only component of these mixtures of which the constitution has recently been definitely established, is β-sitosterol, the main sterol of cottonseed oil [Wallis & Chakravorty, 1937] and of coniferous woods [Sandqvist & Hök, 1930; Sandqvist & Bengtsson, 1931]. This phytosterol differs from cholesterol merely by the presence of an additional C_2H_5 group at C_{24} of the side chain. Its close relationship to stigmasterol, the best known phytosterol, was suggested by the work of Bengtsson [1935], and subsequently Bernstein & Wallis [1937] succeeded in selectively hydrogenating stigmasterol to β-sitosterol, thus justifying the designation of the latter as 22-dihydrostigmasterol.

The recognition that a dietary factor plays an essential role in cholesterol metabolism [Rosenheim & Webster, 1941], made it desirable to investigate the behaviour of a pure sitosterol under controlled conditions on a standard diet and in the presence of the dietary factor. We found that β-sitosterol was excreted unchanged on a sterol-free synthetic diet, just like cholesterol under the same conditions. When administered, however, together with cholesterol-free brain powder or with Thudichum's phrenosin, β-sitosterol is converted into an isomeride of β-sitostanol, for which we propose the name *copro*-sitostanol. In contrast to the original sitosterol, and by analogy with coprosterol, this excretory phytosterol derivative is dextrorotatory. It was characterized by the preparation of various esters and by its oxidation product *copro*-sitostanone.

An interesting relationship was found between the chemical structure of sterols and their metabolism in the presence of the factor. The substitution of a hydrogen atom at C_{24} in the side chain of cholesterol, converting it into β-sitosterol, evidently does not interfere with its metabolism. The introduction of an ethylenic linkage at C_{22}, however, prevents it, as shown by the fact that stigmasterol is excreted unchanged. Ergosterol, containing the same ethylenic linkage, as well as an additional one at C_7 in the ring system, behaves in the same way. It will be recalled that changes in the structure of the side chain exert a profound influence on the conversion of various steroids into antirachitic vitamins.

Experimental

The technique of the animal experiments and the analytical methods used were identical with those described in the preceding communication [Rosenheim & Webster, 1941]. The feeding tests were carried out with a specimen of β-sitosterol from tall oil, M.P. 136–137° (acetate, M.P. 124–125°) as well as with typical sitosterol mixtures obtained from soya beans and wheat germ respectively. The soya bean 'sitosterol', M.P. 138–139°, freed from stigmasterol by bromination of the acetates [Windaus & Hauth, 1906], consisted mainly of γ-sitosterol with a considerable percentage of β-sitosterol, the α-sitosterols having been removed by the preliminary bromination. The wheat germ 'sitosterol', M.P. 138–139°, gave an intense green Tortelli-Jaffé reaction, indicating a high percentage of α-sitosterols in the usual mixture of β- and γ-sitosterols. A 'concentrate' of β-sitosterol was prepared from another specimen of wheat germ sitosterol by means of extensive fractionation and crystallization. The crude sterol mixture (1 kg.) was first crystallized 10 times from absolute alcohol, in batches of 500 g. each, and separated into three main fractions by combining the crystallizations obtained at 36°, 15° and −4° respectively. The top and bottom fractions, containing the γ- and α-sitosterols respectively, were discarded. The middle fractions were acetylated and fractionally recrystallized from benzene-alcohol (1 : 4) as above. After hydrolysis and final recrystallization of the middle fractions, the 'sitosterol', M.P. 138–139°, free from dihydrositosterol and the α-sitosterols, was rich in β-sitosterol but presumably still contained some γ-sitosterol (as indicated by the melting point of the acetate and its optical activity). Specimens of stigmasterol, M.P. 170–171°, and ergosterol, M.P. 162–163°, were also tested. The preparation of cholesterol-free brain powder and 'white matter' has previously been described [Rosenheim & Webster, 1941]. The results of the feeding experiments are given in Table 1.

Table 1

					Sterol intake		Sterol excretion: Total		Sterol excretion: Saturated		
Sterol	Supplement	Per rat, per day g.	No. of rats	Days	Total g.	Per rat, per day mg.	g.	Per rat, per day mg.	g.	Per rat, per day mg.	% of total
β-Sitosterol (tall oil)	Brain powder	1·6	3	7	3·0	143	2·40	114	2·23	106	93
β-Sitosterol (tall oil)	Thudichum's phrenosin	0·3	3	7	3·0	143	2·30	110	1·88	90	82
'Sitosterol' (wheat germ)	Brain powder	1·6	6	7	7·0	167	3·40	95	2·12	59	62
'Sitosterol' (wheat germ)	'White matter'	0·3	3	7	3·4	162	2·81	134	1·78	85	63
β-Sitosterol concentrate (wheat germ)	Brain powder	1·6	6	7	6·0	143	3·79	90	2·76	66	73
β-Sitosterol concentrate (wheat germ)	None	—	6	7	7·0	167	3·47	83	0·55	13	16
'Sitosterol' (soya bean)	Thudichum's phrenosin	0·25	3	7	3·4	162	2·36	112	1·05	50	45
'Sitosterol' (soya bean)	None	—	3	7	3·5	167	2·62	120	0·45	21	18
Stigmasterol	Brain powder	1·6	3	7	2·5	119	1·67	80	0·29	14	18
Ergosterol	Brain powder	1·6	6	7	5·5	132	2·59	60	0·42	10	17

It is evident from these results that hydrogenation of β-sitosterol occurs to the same extent as that of cholesterol, when the diet contains either cholesterol-free brain powder or Thudichum's phrenosin. It may be assumed that the partial conversion into saturated sterols of the sitosterols of soya beans and wheat germ, runs parallel with their content of β-sitosterol, since the fraction of wheat germ sterols, enriched in β-sitosterol by extensive fractionation, shows a markedly increased conversion (73 % of the total faecal sterols) when administered together with brain powder. Further, the saturated sterols obtained in this case yielded the same *copro*-sitostanol as the saturated sterols excreted after feeding with pure β-sitosterol. The remarkable inertness of stigmasterol and ergosterol, both sterols being characterized by the presence of an unsaturated linkage in the side chain, contrasts strongly with the behaviour of β-sitosterol and cholesterol, in which the side chain is saturated.

Copro-*sitostanol*. (*a*) *From β-sitosterol*. The alcoholic solution of the unsaponifiable fraction of the faecal extract was precipitated with a 1 % solution of digitonin after bromination according to Schoenheimer's [1930] method. The saturated digitonides (8 g.) obtained from one of the feeding tests were dissolved in pyridine (80 ml.) and the digitonin precipitated with ether (800 ml.). The ethereal solution of the sterol was freed from pyridine by washing with dilute HCl and water, dried over anhydrous Na_2SO_4 and the crystalline ether residue fractionally recrystallized from 60 vols. of methyl alcohol. The fractions obtained at room temperature and at −4° varied in melting point from 120 to 125°. The higher-melting fractions were combined and yielded on further recrystallization uniform rhombic plates, M.P. 126–127°, $[\alpha]_D + 24{\cdot}6°$; $[\alpha]^{20°}_{5461} + 28{\cdot}4°$ ($c = 1{\cdot}178$ in chloroform, $l = 4$ dm.), $\alpha_{5461}/\alpha_D = 1{\cdot}16$. (Found: C, 83·1; H, 12·4; $C_{29}H_{52}O$ requires C, 83·5; H, 12·8 %.) The melting point and optical activity of the substance remained constant on further recrystallization. From the final mother liquors a small amount (8 mg.) of crystals was obtained, melting indefinitely from 99° to the clearing point 140°, which may have been a mixture of coprosterol and sitostanol (or cholestanol).

In another experiment the alcoholic solution of the 'unsaponifiable' was kept at −4° for some days and deposited sphaerocrystals (M.P. 85–86°, lignoceryl sphingosine?), which adhered firmly to the sides of the flask and were easily separated from the colourless floating crystals of *copro*-sitostanol, M.P. 124–125°. The filtrate was brominated and yielded 6·5 g. of saturated digitonides, from which *copro*-sitostanol was obtained as above.

(*b*) *From wheat germ sitosterol 'concentrate'*. The saturated sterol recovered from the digitonide (1·5 g.) gave *copro*-sitostanol, M.P. 125–126°, $[\alpha]^{20°}_D + 24{\cdot}0°$, melting points of the sterol and its acetate showing no depression when mixed with the specimens obtained from β-sitosterol.

Copro-*sitostanol acetate*, prepared by refluxing *copro*-sitostanol (100 mg.) with acetic anhydride and evaporation of the solvent *in vacuo*, crystallized in shiny leaflets from methyl alcohol, M.P. 88–89°. (Found: C, 80·8; H, 11·5; $C_{31}H_{54}O_2$ requires C, 81·2; H, 11·8 %.) A mixed melting point with coprosterol acetate, M.P. 89–90°, gave a depression of 18–20°.

Copro-*sitostanol benzoate* was obtained by adding benzoyl chloride (0·06 ml.) to a solution of *copro*-sitostanol (100 mg.) in pyridine (1·5 ml.) and pouring the solution into water after 24 hr. The product was recrystallized from alcohol, from which it separated as a mass of needles, M.P. 103–104°. (Found: C, 82·6; H, 10·5; $C_{36}H_{56}O_2$ requires C, 83·0; H, 10·8 %.)

Copro-*sitostanone*. A solution of *copro*-sitostanol (200 mg.) in glacial acetic acid (10 ml.) was mixed with a solution of chromic anhydride (50 mg.) in 90 %

acetic acid (0·5 ml.) and kept at room temperature for 24 hr. On the addition of water crystals deposited which were extracted with ether. The ethereal solution was washed with dilute KOH and water, the ether removed after drying over anhydrous Na_2SO_4 and the crystalline residue (190 mg.) recrystallized from methyl alcohol. The ketone crystallized in thin plates, M.P. 113–114°. (Found: C, 83·3; H, 11·8; $C_{29}H_{50}O$ requires C, 83·5; H, 12·2%) and was characterized by its *o*-tolylsemicarbazone. This derivative was obtained by dissolving the ketone (20 mg.) and *o*-tolylsemicarbazide (20 mg.) in alcohol (2 ml.) containing a drop of acetic acid. Copro-*sitostanone*-o-*tolylsemicarbazone* crystallizes in colourless needles, M.P. 198–199°, from benzene alcohol.

After the isolation of *copro*-sitostanol from rats' faeces (early in 1937) a communication appeared by Marker & Wittle [1937], in which the preparation of 24-ethylcoprostanol, M.P. 127°, from β-sitostenone by the usual chemical methods was described. It may be assumed that this substance is identical with the natural excretory product, for which the name *copro*-sitostanol seems more appropriate.

In the control feeding experiments in which β-sitosterol was added to the standard diet without supplements of brain powder etc. the alcoholic solutions of the 'unsaponifiable' deposited the bulk of the sterols on cooling to −4° in characteristic crystals. A single recrystallization sufficed to yield β-sitosterol, M.P. 136–137°, a mixed melting point with the original sterol showing no depression. In these cases, as well as in the experiments with 'sitosterols' of soya beans and wheat germ, we were unable to isolate any saturated sterols by crystallization, although the analytical figures indicated the presence of traces of saturated sterols.

Stigmasterol, M.P. 170–171°, and ergosterol, M.P. 162–163°, not depressed in admixture with respective authentic specimens, were obtained by direct crystallization as above from the faeces after feeding the sterols mixed with the standard diet together with, or in the absence of, brain powder.

Summary

The metabolism and excretion of β-sitosterol is influenced by the diet and dependent on the presence of a dietary factor.

When administered together with cholesterol-free brain powder or Thudichum's phrenosin, β-sitosterol is hydrogenated and excreted as *copro*-sitostanol, which has been characterized by various derivatives.

The relationship between chemical structure of sterols and their absorption and excretion is discussed.

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103. SHEEP BODY FATS

1. COMPONENT ACIDS OF FATS FROM ANIMALS FED ON HIGH AND LOW PLANES OF NUTRITION

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In 1938 an investigation on the effects of different planes of nutrition of ewes upon the birth weight and growth of lambs was undertaken at the Animal Nutrition Institute of the School of Agriculture, Cambridge, by Dr Verges, with the result that two groups of ewes respectively of about 70 and 150 lb. live weight were obtained from the restricted and full diets given. Five of the 70 lb. animals were then fed on a high plane of nutrition ('supermaintenance ration'), whilst five of the 150 lb. ewes were given a restricted diet ('submaintenance ration'), an animal from each group being killed at intervals until, finally, the first group attained 150 lb. live weight, whilst the second group had been brought down to about 70 lb. live weight. Dr J. Hammond suggested that the fat from these animals would repay study since throughout the experiment they had been on standard and known quantities of food. In this and the following communication some account is given of the component acids and glycerides in the perinephric and external tissue fats of some of the ewes. The opportunity to study sheep body fats from animals of known history was especially welcomed because all previous detailed information as to the acids and glycerides present referred to specimens of market origin (mutton tallow); the analyses now recorded afford data for sheep fats parallel with those already obtained for ox and pig depot fats from animals reared on specific diets.

Experimental

The 'supermaintenance rations' fed to the ewes of 70 lb. live weight consisted of lucerne hay of medium quality *ad lib.* together with 4–5 lb. daily of a concentrate mixture consisting of 1 part by weight each of locust bean meal and white fish meal, 2 parts by weight each of broad bran, flaked maize, linseed cake and split peas, and 3 parts by weight of crushed oats. The 'submaintenance ration' fed to the ewes of 150 lb. live weight consisted of wheat straw *ad lib.* until the live weight of the animal fell to 90 lb., and subsequently of $\frac{3}{4}$ lb. of wheat straw only each day.

Some general data for the animals at the time of slaughter are quoted in Table 1.

The component acids of the perinephric and external tissue fats of three ewes from each group (nos. 26, 12 and 4, and nos. 30, 9 and 21) have been examined in detail. (The quantity of perinephric fat available from the control animal no. 11 of 70 lb. live weight was too small to permit detailed analysis.) The yield of fat extracted by acetone from the tissues is shown in Table 2.

Table 1

Ewe no.	Days on special ration	Live weight lb.	Fatty tissues: Perinephric g.	Fatty tissues: External g.
	(*a*) Ewes on 'supermaintenance' ration			
11	0	70	27	223
26	22	91	138	671
20	38	105	253	1377
12	58	127	703	3939
4	81	151	2026	5580
	(*b*) Ewes on 'submaintenance' ration			
30	0	148	1019	7110
29	13	131	1441	5100
9	100	113	406	1218
10	147	91	465	1615
21	209	72	341	742

Table 2. *Composition of ewe fatty tissues*

Ewe no.	Live wt. at death lb.	Perinephric tissue: Fat %	Perinephric tissue: Fat I.V.	Perinephric tissue: Connective tissue %	Perinephric tissue: Water, etc. (by diff.) %	External tissue: Fat %	External tissue: Fat I.V.	External tissue: Connective tissue %	External tissue: Water, etc. (by diff.) %
		(*a*) Ewes on 'supermaintenance' rations							
26	91	90·3	48·2	6·0	3·7	44·6	52·0	18·8	36·6
12	127	96·7	43·4	2·7	0·6	80·8	49·1	8·2	11·0
4	151	97·5	42·7	1·6	0·9	83·6	50·2	8·0	8·4
		(*b*) Ewes on 'submaintenance' rations							
30	148	91·6	41·0	1·1	7·3	87·6	47·9	3·4	9·0
9	113	98·0	40·2	1·3	0·7	82·0	44·6	8·3	9·7
21	72	97·1	34·3	1·6	1·3	69·9	44·3	12·9	17·2

The proportion of fat in the external tissues of the ewe (no. 21) which had been reduced to 72 lb. live weight, although lower than in those of the fat animals, was considerably greater than that in the external tissues of the ewe (no. 26) of 91 lb. live weight (which had been brought up on a restricted diet until 22 days before it was killed). The general unsaturation of the fats, as indicated by their iodine values, declined somewhat in both the 'supermaintenance' and 'submaintenance' groups as the experiments proceeded.

The component acids of the selected fats were determined by means of the procedure followed in recent similar work in this laboratory [Hilditch & Longenecker, 1937; Hilditch *et al.* 1939]: the mixed fatty acids from hydrolysis of 130–150 g. of each fat were separated into 'solid' and 'liquid' acids by the different solubilities of the lead salts in alcohol, and thereafter the methyl esters of each group of acids were fractionally distilled through an electrically heated and packed column at low pressure (0·1–0·2 mm.).

Considerations of space lead us to confine our data here to the final weight and molecular percentages observed for the acids in each of the fats (Table 3); but it should be mentioned that the unsaturation of the methyl esters of C_{18} acids from the 'solid' acids was uniformly large (I.V. from 13 to 25), presumably owing to the presence of small proportions of vaccenic acid [Bertram, 1928], a solid acid isomeric with oleic acid.

Table 3. *Component acids of perinephric and external tissue fats of ewes*

	'Supermaintenance' group			'Submaintenance' group			
Ewe no.	26	12	4	30	9	21	
Live wt. (lb.)	91	127	151	148	113	72	
Acid	(*a*) Weight % (excluding unsaponifiable matter)						Mean
	Perinephric fats						
Lauric	—	—	—	0·1	0·1	—	Trace
Myristic	2·9	2·7	2·3	1·7	3·0	2·8	2·6
Palmitic	24·0	24·7	26·2	26·8	23·6	23·0	24·7
Stearic	24·9	28·3	27·1	30·1	31·7	37·8	30·0
Tetradecenoic	0·7	0·3	0·3	0·2	0·2	0·2	0·3
Hexadecenoic	2·4	0·9	1·0	0·9	1·3	1·0	1·2
Oleic	39·2	36·8	38·7	34·8	35·4	32·1	36·2
Octadecadienoic	5·2	5·7	3·3	4·3	3·9	2·2	4·1
Unsaturated C_{20-22}	0·7	0·6	1·1	1·1	0·8	0·9	0·9
	External tissue fats						
Lauric	0·4	0·6	0·9	0·7	0·6	0·3	0·6
Myristic	3·4	3·0	3·1	1·9	2·2	3·7	2·9
Palmitic	27·8	28·0	28·3	33·9	30·5	24·3	28·8
Stearic	14·7	16·2	13·5	15·3	20·1	24·6	17·4
Tetradecenoic	0·4	0·3	0·2	0·3	0·3	0·3	0·3
Hexadecenoic	1·6	0·8	0·6	0·9	1·2	0·7	1·0
Oleic	46·3	46·6	50·8	41·2	41·4	44·1	45·0
Octadecadienoic	4·8	3·9	1·9	4·9	2·8	1·5	3·3
Unsaturated C_{20-22}	0·6	0·6	0·7	0·9	0·9	0·5	0·7
	(*b*) Molar % (excluding unsaponifiable matter)						
	Perinephric fats						
Lauric	—	—	—	0·2	0·2	—	0·1
Myristic	3·5	3·2	2·8	2·1	3·6	3·3	3·1
Palmitic	25·6	26·4	28·0	28·6	25·3	24·7	26·4
Stearic	23·9	27·3	26·1	29·0	30·5	36·6	28·9
Tetradecenoic	0·8	0·3	0·3	0·3	0·3	0·2	0·4
Hexadecenoic	2·6	1·0	1·1	1·0	1·4	1·1	1·4
Oleic	38·0	35·7	37·5	33·7	34·3	31·2	35·0
Octadecadienoic	5·0	5·6	3·2	4·2	3·8	2·1	4·0
Unsaturated C_{20-22}	0·6	0·5	1·0	0·9	0·6	0·8	0·7
	External tissue fats						
Lauric	0·5	0·8	1·2	1·0	0·8	0·4	0·8
Myristic	4·1	3·6	3·7	2·3	2·7	4·4	3·5
Palmitic	29·4	29·7	29·9	35·8	32·3	25·9	30·5
Stearic	14·1	15·6	12·9	14·5	19·2	23·6	16·6
Tetradecenoic	0·5	0·3	0·2	0·4	0·3	0·4	0·3
Hexadecenoic	1·7	0·8	0·7	1·0	1·3	0·8	1·1
Oleic	44·6	44·9	48·9	39·5	40·0	42·5	43·4
Octadecadienoic	4·6	3·8	1·9	4·7	2·7	1·5	3·2
Unsaturated C_{20-22}	0·5	0·5	0·6	0·8	0·7	0·5	0·6

Discussion

Only three detailed analyses of sheep depot fats (mutton tallows) have been recorded previously, and these do not take account of unsaturated minor component acids other than octadecadienoic; the earlier data are collected in Table 4.

These older figures fall for the most part within the range of those obtained in the course of our present work, and it would appear that, whilst the specimen examined by Collin *et al.* [1929] was wholly perinephric fat, those described by Armstrong & Allan [1924] may have been mixed body fats in which external tissue fat was also present.

Table 4. *Earlier analyses of sheep depot fats*

Acid (weight %)	Armstrong & Allan [1924] South American	Armstrong & Allan [1924] Australian	Collin *et al.* [1929] Australian
Myristic	1	2	4
Palmitic	21	25	25
Stearic	30	23	31
Oleic	43	47	36
Octadecadienoic	5	3	4

From the whole series of sheep fats which we have now studied, it appears that, in the perinephric fats, the molar content of palmitic (+myristic) acid is about $26{\cdot}5 \pm 2\%$ and is thus somewhat lower than the value ($30 \pm 3\%$) which Hilditch & Longenecker [1937] have shown to be characteristic for ox depot fats, and Hilditch *et al.* [1939] for pig depot fats. The molar palmitic acid content of the external tissue fats is distinctly higher (mean value $30{\cdot}5\%$) and is also more variable, values from $25{\cdot}9$ to $35{\cdot}8\%$ (mol.) being observed. It is notable that, in ox and pig depot fats, those from the external tissues contain somewhat less palmitic acid than the more saturated perinephric fats, but that in the sheep the external tissue fats, although as usual less saturated than the perinephric fats, nevertheless possess higher proportions of combined palmitic acid than the latter.

Of the minor component saturated acids, myristic acid is present to about the same extent as in ox depot fats, but arachidic acid was not definitely detected. In the unsaturated series, hexadecenoic acid (which is usually present to the extent of 2–3% in ox and pig depot fats) only forms about 1% of the total fatty acids, whilst the proportion of unsaturated C_{20-22} acids is similar and thus probably intermediate between that found in ox and in pig fats. Polyethenoid C_{18} acids approach in quantity those in pig depot fats and are definitely more prominent than in ox depot fats; the proportion, although somewhat variable, is usually of the order of $4 \pm 1\%$ (mol.) of the total fatty acids. Acids from ester-fractions consisting substantially of the unsaturated C_{18} group yielded, on addition of bromine, traces of ether-insoluble bromo-additive products which darkened at 165–170° without melting and of petrol-insoluble products which melted at 114°; oxidation of their potassium salts with cold dilute permanganate solution likewise yielded only minute amounts of tetrahydroxystearic acids, M.P. 172°, and M.P. 153–154° (accompanying large quantities of dihydroxystearic acid, M.P. 130°, from the oleic acid also present). Apart from possible traces of polyethenoid C_{18} acids, the octadecadienoic acid of the sheep fats is different from ordinary or seed-fat linoleic acid, although traces of the latter are also present. The sheep fats thus closely resemble ox depot and cow milk fats, and many animal liver fats, in containing small quantities of an octadecadienoic acid not identical with seed-fat linoleic acid.

It is interesting to compare (Table 5) the total molar proportions of C_{16} and C_{18} acids in the sheep fats (for this purpose including minor component C_{14} and C_{12} acids with the C_{16} group, and the minor proportions of C_{20} and C_{22} acids with the C_{18} acids).

The approximate constancy of the total C_{16} and C_{18} groups of acids within each series of depot fats is as well-marked as in ox or pig depot fats previously studied in this laboratory [Banks & Hilditch, 1931; 1932; Dean & Hilditch, 1933; Hilditch & Longenecker, 1937]. In the six perinephric fats, the C_{18} (stearic, oleic, octadecadienoic) acids amount to 68–70 mols. per 100 mols. of

Table 5. *Molar percentages of C_{16} and C_{18} groups of acids*

	'Supermaintenance' group			'Submaintenance' group			
Ewe no. ...	26	12	4	30	9	21	
Live wt. (lb.) ...	91	127	151	148	113	72	
	Perinephric fats						Mean
C_{16} (+C_{14}, C_{12}) acids	32·5	30·9	32·2	32·2	30·8	29·3	31·4
C_{18} (+C_{20-22}) acids	67·5	69·1	67·8	67·8	69·2	70·7	68·6
	External tissue fats						
C_{16} (+C_{14}, C_{12}) acids	36·2	35·2	35·7	40·5	37·4	31·9	36·2
C_{18} (+C_{20-22}) acids	63·8	64·8	64·3	59·5	62·6	68·1	63·8

total fatty acids, although the stearic acid content, for example, varies from 23·9 to 36·6% (mol.); in the external tissue fats which, with one exception, contain more C_{16} acids than the perinephric fats, the total C_{18} acids form from 59·5 to 64·8% (mol.) of the total fatty acids, whilst the stearic acid content varies from 12·9 to 19·2% (mol.). These relationships are exactly similar to those observed in depot fats of oxen or pigs and, as will be seen from the communication which follows [Hilditch & Zaky, 1941], are consequent upon the characteristic glyceride structure which, as depot fats rich in stearic acid, sheep fats share with ox, etc. depot fats.

Up to this point, the twelve fats under investigation have been discussed as a whole, and as typical of depot fats produced in lean or fat sheep which have received diets of normal, though differing, character. Some interesting features are revealed, however, by consideration of the separate dietary experiments in which on the one hand, some of the animals were given a fattening ration, whilst on the other hand others were depleted in fat by feeding on a low plane of nutrition.

Fat deposited by ewes fed on 'supermaintenance' rations

From the weight of fat in the depots of each animal and the weight percentages of the component fatty acids (Table 3) it is possible to obtain a rough estimate of the weight of each fatty acid present in the depots of the control animal (91 lb. live weight) and of the animals killed respectively after 58 and 81 days' intensive feeding at respective live weights of 127 and 151 lb. (Table 6).

Table 6. *Total amounts of fatty acids in sheep depot fats (g.)*

	Perinephric fats			External tissue fats		
Ewe no. ...	26	12	4	26	12	4
Fatty tissue (g.)	138	703	2026	671	3939	5580
% fat	90·3	96·7	97·5	44·6	80·8	83·6
Fat (g.)	125	680	1975	299	3183	4664
Fatty acids (g.)	119	650	1887	286	3041	4456
Lauric	—	—	—	1	18	40
Myristic	3	17	43	10	91	138
Palmitic	29	161	494	79	852	1261
Stearic	30	184	512	42	493	601
Tetradecenoic	1	2	6	1	9	9
Hexadecenoic	3	6	19	5	24	27
Oleic	46	239	730	132	1417	2264
Octadecadienoic	6	37	62	14	119	85
Unsaturated C_{20-22}	1	4	21	2	18	31

It is permissible to take the data for ewe no. 26 as an approximate measure of the quantities of fatty acids already present in the depots of the other animals

at the outset of the experiment, although there are indications that the ewes varied somewhat in total fat content at corresponding stages of the experiment. Nevertheless, the quantities of each acid at the commencement are small in comparison with those added during fattening, and any error thus introduced will not be serious. We thus arrive at an estimate of the percentage (weight) composition of the fatty acids deposited as depot fats in ewes nos. 12 and 4 (Table 7).

Table 7. *Estimated proportions (weight %) of fatty acids deposited by ewes nos. 12 and 4*

	Perinephric fats		External tissue fats	
Ewe no.	12	4	12	4
Approx. fat deposited (g.) ...	555	1850	2884	4365
Fatty acids (wt. %):				
Lauric	—	—	0·6	0·9
Myristic	2·6	2·3	2·9	3·1
Palmitic	24·9	26·3	28·1	28·4
Stearic	29·0	27·3	16·4	13·4
Tetradecenoic	0·2	0·3	0·3	0·2
Hexadecenoic	0·6	0·9	0·7	0·5
Oleic	36·3	38·6	46·6	51·1
Octadecadienoic	5·8	3·2	3·8	1·7
Unsaturated C_{20-22}	0·6	1·1	0·6	0·7

The figures in Table 7 are of interest from several points of view. They suggest that the close constancy in the contents of the C_{16} and C_{18} groups of acids is maintained throughout the whole period of fattening, with the characteristically slight differences maintained in each group as between the perinephric and the external tissue fats. There is also definite indication that in the earlier stages of fattening somewhat more stearic and octadecadienoic acids (and correspondingly less oleic acid) are produced than in the later stages; indeed, the figures suggest the possibility that in the final stages some of the octadecadienoic acid previously deposited in the external tissues has disappeared either by mobilization or by conversion into oleic glycerides. At the same time the close constancy of the combined proportions of oleic, stearic and octadecadienoic acids may be remarked (71·1 and 69·1 % in the added perinephric and 66·8 and 66·2 % in the added external tissue fats). It may reasonably be concluded that the proportions of component acids shown in Table 7 are specific for fat deposition by sheep on a normal fattening diet.

Fat lost by ewes fed on 'submaintenance' rations

Table 8 shows the approximate weight of each fatty acid in the depots of the control animal (148 lb. live weight) and of the ewes killed respectively after 100 and 209 days' restricted feeding when the respective live weights were 113 and 72 lb.

Taking the data for ewe no. 30 as an approximate measure of the amounts of each fatty acid present in the depots of nos. 9 and 21 at the commencement of restricted diet (as in the case of the intensive feeding experiment above), an estimate can be made of the percentage (weight) proportions of each fatty acid removed from the fat depots in ewes nos. 9 and 21 during their maintenance on the restricted diet (Table 9).

Table 8. *Total amounts of fatty acids in sheep depot fats (g.)*

	Perinephric fats			External tissue fats		
Ewe no. ...	30	9	21	30	9	21
Fatty tissue (g.)	1019	406	341	7110	1218	742
% fat	91·6	98·0	97·1	87·6	82·0	69·9
Fat (g.)	932	398	331	6230	999	519
Fatty acids (g.)	891	380	316	5950	954	496
Lauric	1	Trace	—	42	6	2
Myristic	15	11	9	113	21	18
Palmitic	239	90	73	2017	291	121
Stearic	268	121	119	911	192	122
Tetradecenoic	2	1	1	18	3	2
Hexadecenoic	8	5	3	54	11	3
Oleic	310	134	101	2450	395	219
Octadecenoic	38	15	7	291	27	7
Unsaturated C_{20-22}	10	3	3	54	8	2

Table 9. *Estimated proportions (weight %) of fatty acids mobilized from depots of ewes nos. 9 and 21*

	Perinephric fats		External tissue fats	
Ewe no. ...	9	21	9	21
Approx. fat lost (g.)	534	601	5231	5711
Fatty acids (wt. %):				
Lauric	0·2	0·2	0·7	0·7
Myristic	0·8	1·0	1·8	1·8
Palmitic	29·1	28·9	34·5	34·8
Stearic	28·8	25·9	14·4	14·5
Tetradecenoic	0·2	0·2	0·3	0·3
Hexadecenoic	0·6	0·9	0·9	0·9
Oleic	34·4	36·3	41·2	40·9
Octadecadienoic	4·5	5·4	5·3	5·2
Unsaturated C_{20-22}	1·4	1·2	0·9	0·9

The figures in Table 9 should be compared with the corresponding data in Table 7 (referring to the component acids of fat which is being added to the depots). In the perinephric fats of the group on restricted diet, the proportions of the fatty acids in the mobilized fat are on the whole similar to those added by the animals which were intensively fed. Some slight differences are however observable, and these are much more accentuated in the external tissue fats. The proportion of palmitic acid in the mobilized fat is slightly higher than in the deposited fats (Table 7), whilst that of oleic and stearic acids is correspondingly somewhat lower. Thus palmitic glycerides have been, to a slight degree, preferentially mobilized as compared with oleo- or stearo-glycerides.

In the external tissue fats this tendency is more marked. It has already been noted (p. 935) that these possess higher proportions of combined palmitic acid than the sheep perinephric fats, and the proportion of palmitic acid deposited in the depot fats during intensive feeding (Table 7) is also somewhat greater in the external tissues than in the perinephric fats. Similarly, during maintenance of restricted diet, the fat withdrawn from the external tissues is definitely richer in combined palmitic acid, and poorer in oleic and (to a less degree) stearic acid, than that deposited during intensive feeding.

This general tendency towards preferential mobilization of palmito-glycerides in both groups of sheep fats may be further illustrated by noting the united proportions of C_{18} (oleic, stearic and octadecadienoic) acids removed from the depots: 67·7 and 67·6% in the cases of the perinephric fats, and 60·9 and 60·6%

in the external tissue fats. The proportions in the perinephric fats are only slightly lower than those of the added C_{18} acids in the 'supermaintenance' experiments (71·1 and 69·1%), but those in the fats removed from the external tissues are considerably lower than the C_{18} acids deposited during full feeding (66·8 and 66·2%). These results seem to differ materially from those obtained from the fats of pigs which had been fasted for prolonged periods [Hilditch & Pedelty, 1940]. In the pig fats, although no wide degree of selectivity in the mobilization of any one fatty acid component of the depot glycerides was detected, it was clear that oleic glycerides were removed to a greater extent than those of the saturated acids; whereas in the sheep fats palmitic acid has consistently been shown to be somewhat more freely mobilized than the other major components. Again, there is definite reluctance in the pig to mobilize the minor proportions of octadecadienoic and unsaturated C_{20-22} glycerides present, but in the sheep the glycerides of these acids appear to be removed at least as readily as the oleic glycerides. The conditions in the two series of experiments were, however, not identical: the pigs were fasted and only received water during the trials, whilst the sheep were given a restricted amount of wheat straw. It is therefore not certain whether the observed difference in the nature of the fat withdrawn is specific for the animals concerned, or whether it may have been influenced by the different dietary conditions.

Attention may again be directed to the circumstance that practically the whole of these depot fats consists of mixed glycerides (in which palmitodioleins and oleopalmitostearins are most abundant) and that therefore any tendency towards withdrawal of a particular fatty acid is partly obscured by the fact that other acids present in the mixed glycerides are inevitably concurrently involved.

We desire to thank Drs Hammond and Verges for the opportunity to study the valuable experimental material available from their field trials. We are also indebted to the Superintendent of the Low Temperature Station, Cambridge, for assistance in collecting and storing the fatty tissues; and to the Department of Scientific and Industrial Research for permission to publish the results of this work, which was carried out as part of the programme of the Food Investigation Board.

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104. SHEEP BODY FATS

2. COMPONENT GLYCERIDES OF PERINEPHRIC AND EXTERNAL TISSUE FATS FROM THE SAME ANIMAL

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The glycerides present in the perinephric and external tissue fats of a sheep have been studied by means of the procedure described in detail in the case of an ox depot fat [Hilditch & Paul, 1938], and later applied to the perinephric and outer back fats from a pig by Hilditch & Pedelty [1940]. For the present purpose the fats chosen for investigation were from the animal (ewe no. 4) referred to in the preceding communication [Hilditch & Pedelty, 1941], since this sheep, which had been reared from 70 to 150 lb. live weight on the 'super-maintenance' ration (cf. p. 932), furnished the comparatively large quantities of fat required for work of this kind.

Experimental

The essential data were obtained by methods which were described in full in connexion with ox depot fat glycerides [Hilditch & Paul, 1938] and also summarized in the subsequent communication on pig depot fats [Hilditch & Pedelty, 1940]. Description of the procedure may therefore here be confined to a brief recapitulation of the various stages.

(*a*) The sheep fats (perinephric 750 g., and external tissue fat 580 g.) were each systematically crystallized from acetone (at 0° or room temperature, at various concentrations and for varying periods), with the result that a number of fractions were obtained and ultimately grouped, when sufficiently similar in iodine value, into three main fractions in the case of each fat.

(*b*) The component acids in each fraction were determined by ester-fractionation.

(*c*) The amounts of fully saturated glycerides present in the fractions more sparingly soluble in acetone (I.V. below 40) were determined by oxidation of a portion of each fraction with $KMnO_4$ in acetone solution. When sufficient fully saturated glycerides (10 g. or more) were available, their component acids were determined by ester-fractionation; otherwise, the latter were calculated as mixtures of palmitic and stearic acids from the observed equivalent of the fully saturated glycerides.

(*d*) In the fractions most soluble in acetone (in which any tri-unsaturated glycerides and most of the di-unsaturated glycerides are concentrated), the amount of tri-C_{18} glycerides was determined, in the form of tristearin, by hydrogenating a portion as completely as possible and separating the hydrogenated product, by crystallization from anhydrous ether, into a series of fractions. The equivalents of the fractions obtained were determined, and the component acids of the least soluble fraction obtained by ester-fractionation and

calculated as a mixture of tristearin with palmitodistearin. Small proportions of incompletely hydrogenated material passed with the more soluble fully saturated glycerides into the fractions relatively soluble in ether, and were calculated as oleodistearin from the iodine values of the fractions concerned.

In view of recent findings [Atherton & Hilditch, 1941] that migration of acyl groups between triglyceride molecules occurs slowly, and to a small extent, during hydrogenation at 170–180°, the hydrogenations were conducted as far as possible at 95° by means of Raney nickel catalyst, and completed by a short treatment at 170° after addition of a small quantity of active nickel on kieselguhr.

The whole of the data obtained as described above permit the component glycerides in each fraction to be grouped, according to the proportions of palmitic, stearic and unsaturated C_{18} acids present, into (*a*) dipalmitomono-C_{18} glycerides, palmitodi-C_{18} glycerides, and tri-C_{18} glycerides, and (*b*) fully saturated, mono-unsaturated-disaturated, di-unsaturated-monosaturated and tri-unsaturated glycerides. From the proportions of these various classes, the individual components in each fraction of the original fat can be deduced within fairly close limits. (The minor component acids, for this purpose, have to be included with appropriate major components: lauric, myristic, tetradecenoic and hexadecenoic acids are included with palmitic, arachidic with stearic, and octadecadienoic and unsaturated C_{20-22} acids with oleic acid. The minor component acids of the sheep fats, other than octadecadienoic (2–3%), together formed 5–6% of the total fatty acids, so that probably about 15% of the glycerides contained one of the minor component acyl groups.)

The component acids of the two fats, determined on the total fatty acids of each fat (preceding paper, p. 933), were found to be as below:

	Perinephric fat		External tissue fat	
Acid	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Lauric	—	—	0·9	1·2
Myristic	2·3	2·8	3·1	3·7
Palmitic	26·2	28·0	28·3	29·9
Stearic	27·1	26·1	13·5	12·9
Tetradecenoic	0·3	0·3	0·2	0·2
Hexadecenoic	1·0	1·1	0·6	0·7
Oleic	38·7	37·5	50·8	48·9
Octadecadienoic	3·3	3·2	1·9	1·9
Unsaturated C_{20-22}	1·1	1·0	0·7	0·6

Glyceride structure of the perinephric fat

Systematic crystallization from acetone finally gave four fractions which were grouped for analysis into three fractions A, B, C as follows:

	Fractions isolated	
	g.	I.V.
A	255·2	19·3
B	188·8	39·5
C	53·1	61·5
	250·7	65·0

The proportions, characteristics and component acids of the three fractions are given in Table 1.

Table 1. *Fractions (A, B, C) separated by crystallization of the perinephric fat (747·8 g.) from acetone*

	A		B		C	
Wt. of fraction (g.)	255·2		188·8		303·8	
I.V.	19·3		39·5		64·4	
Sap. equiv.	286·6		288·6		285·9	
Glycerides % (wt.)	34·1		25·3		40·6	
Glycerides % (mol.)	34·2		25·1		40·7	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:						
Lauric	—	—	—	—	0·5	0·6
Myristic	1·8	2·1	1·0	1·1	3·4	4·1
Palmitic	29·1	31·2	23·5	25·4	23·1	24·5
Stearic	44·4	42·8	30·4	29·5	9·3	8·9
Arachidic	2·6	2·3	2·6	2·3	0·3	0·3
Tetradecenoic	0·2	0·2	0·2	0·3	0·5	0·5
Hexadecenoic	1·2	1·3	1·1	1·2	2·1	2·3
Oleic	20·7	20·1	39·4	38·5	52·3	50·5
Octadecadienoic	—	—	0·8	0·8	7·9	7·7
Unsaturated C_{20-22}	—	—	1·0	0·9	0·6	0·6

The fully saturated glycerides in the less soluble fractions A and B were determined, together with their component acids, with the results given in Table 2. (As a precaution, the most soluble fraction C was similarly examined; but 50 g. yielded, after two oxidations, only 0·3 g. of neutral material which still possessed I.V. 10·3. Fully saturated glycerides were thus completely absent from fraction C.)

Table 2. *Fully saturated glycerides present in fractions A and B of the perinephric fat*

	A*				B	
Wt. of fraction oxidized (g.)	100·5				100·0	
	(i)		(ii)			
Wt. of crude F.S.G. (g.)	27·2		15·5		4·0	
I.V. of crude F.S.G.	0·3		0·8		0·7	
Sap. equiv. of crude F.S.G.	288·0		281·4		274·9	
Acid value of crude F.S.G.	1·7		1·8		1·7	
% (wt.)	25·0		14·0		3·8	
% (mol.)	24·9		14·4		4·0	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:						
Myristic	—	—	13·8	16·1	—	—
Palmitic	30·1	32·3	36·0	37·4	77·1	78·9
Stearic	69·0	66·9	46·7	43·6	22·9	21·1
Arachidic	0·9	0·8	3·5	2·9	—	—

* The crude fully saturated glycerides from fraction A were separated into two groups by crystallization from ether before detailed analysis.

In the most soluble fraction C the tri-C_{18} glycerides were estimated by the hydrogenation and crystallization procedure described above. The hydrogenated product (*ca.* 80 g.) yielded the following fractions when crystallized from anhydrous ether:

	Least soluble				Most soluble
Wt. (g.)	32·9	19·2	3·1	3·1	18·8
I.V.	0·0	0·0	0·9	2·1	2·2
Sap. equiv.	290·6	287·5	282·1	282·0	284·0
% (wt.)	42·7	24·8	4·1	4·0	24·4
% (mol.)	42·2	24·8	4·2	4·1	24·7

The component acids in the least soluble portion were found (by ester-fractionation) to be palmitic 22·4, stearic 76·6, and C_{20-22} 1·0% (mol.), from which it was estimated to contain 32·8% (mol.) of tri-C_{18} glycerides (including the C_{20-22} group), corresponding to 13·9% (mol.) of tri-C_{18} glycerides in fraction C. The small I.V. of the more soluble fractions, reckoned as oleodistearin, amounted to 2·2% (mol.) of the fraction C. The total tri-C_{18} glycerides in fraction C thus amounted to 16·1% (mol.), corresponding to 6·5% (mol.) in the whole perinephric fat.

Table 3. *Probable component glycerides (% mol.) present in the constituent fractions and in the whole ewe perinephric fat*

	A		B		C	Whole fat
Glycerides	34·2		25·1		40·7	100·0
Component acids (increments):						
Lauric	—		—		0·3	0·3
Myristic	0·7		0·3		1·7	2·7
Palmitic	10·7		6·4		10·0	27·1
Stearic	14·6		7·4		3·6	25·6
Arachidic	0·8		0·6		0·1	1·5
Tetradecenoic	0·1		0·1		0·2	0·4
Hexadecenoic	0·4		0·3		0·9	1·6
Oleic	6·9		9·6		20·6	37·1
Octadecadienoic	—		0·2		3·1	3·3
Unsaturated C_{20-22}	—		0·2		0·2	0·4
	F.S.G. (13·4%)	Mixed (20·8%)	F.S.G. (1·0%)	Mixed (24·1%)		
Groups of component acids (increments):						
C_{16} (+C_{14}, C_{12})	5·4	6·5	0·8	6·3	13·1	32·1
Stearic (+C_{20})	8·0	7·4	0·2	7·8	3·7	27·1
'Oleic' (+C_{20-22})	—	6·9	—	10·0	23·9	40·8
Groups of component glycerides (increments):						
Tripalmitin	—	—	0·4	—	—	0·4
Dipalmitomono-C_{18}	2·9	1·5	0·6	—	5·1	10·1
Palmitodi-C_{18}	10·2	16·5	—	18·9	29·1	74·7
Tri-C_{18}	0·3	2·8	—	5·2	6·5	14·8
Fully saturated	13·4	—	1·0	—	—	14·4
Mono-'oleo'-disaturated	—	19·4	—	18·1	9·7–16·3	47·2–53·8
Di-'oleo'-monosaturated	—	1·4	—	6·0	31·0–17·9	38·4–25·3
Tri-'olein'	—	—	—	—	Nil–6·5	Nil–6·5
	Probable component glycerides					
Fully saturated (14·4%):						
Tripalmitin	—	—	0·4	—	—	0·4
Dipalmitostearin	2·9	—	0·6	—	—	3·5
Palmitodistearin	10·2	—	—	—	—	10·2
Tristearin	0·3	—	—	—	—	0·3
Mono-'oleo'-disaturated (47·2–53·8%):						
Hexadecenopalmitostearin	—	1·5	—	—	—	1·5
'Oleo'-dipalmitin	—	—	—	—	5·1	5·1
'Oleo'-palmitostearin	—	16·5	—	18·1–12·9	4·6–11·2	39·2–40·6
'Oleo'-distearin	—	1·4	—	Nil–5·2	—	1·4–6·6
Di-'oleo'-monosaturated (38·4–25·3%):						
Palmitodi-'olein'	—	—	—	0·8–6·0	24·5–17·9	25·3–23·9
Stearodi-'olein'	—	1·4	—	5·2–Nil	6·5–Nil	13·1–1·4
Tri-'olein' (Nil–6·5%)	—	—	—	—	Nil–6·5	Nil–6·5

Applying the methods of interpretation described in the earlier communications cited to the foregoing data, we arrive at the general statement of probable component glycerides in the ewe perinephric fat given in Table 3. Where alternative possibilities arise, the results are given, as usual, in the form of limiting values for 'oleo'-palmitostearin, palmitodi-'olein', stearodi-'olein' and tri-'olein'.[1]

Glyceride structure of the external tissue fat

Systematic crystallization from acetone yielded eight portions which were finally grouped for analysis into three sets, A, B, C, as follows:

Fractions isolated		Fractions studied		
g.	I.V.	No.	g.	I.V.
102·8	26·7	A	179·7	29·7
25·2	33·8			
51·7	35·6			
20·9	45·0	B	29·2	45·4
8·3	46·5			
18·2	54·0	C	370·8	59·7
108·0	58·7			
244·6	61·9			

The proportions, characteristics and component acids of the three composite portions A, B, C, are given in Table 4.

Table 4. *Fractions (A, B, C) separated by crystallization of the external tissue fat from acetone*

	A		B		C	
Wt. of fraction (g.)	179·7		29·2		370·8	
I.V.	29·7		45·4		59·7	
Sap. equiv.	285·4		285·9		285·5	
Glycerides % (wt.)	31·0		5·0		64·0	
Glycerides % (mol.)	31·0		5·0		64·0	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:						
Myristic	2·6	3·1	1·9	2·3	3·6	4·3
Palmitic	31·0	32·9	29·9	31·7	29·0	30·8
Stearic	30·7	29·4	17·5	16·7	7·5	7·2
Arachidic	0·5	0·5	—	—	0·4	0·4
Tetradecenoic	Trace	Trace	—	—	0·2	0·3
Hexadecenoic	0·8	0·9	3·3	3·5	0·8	0·8
Oleic	34·4	33·2	46·5	44·9	53·9	51·9
Octadecadienoic	—	—	0·9	0·9	3·2	3·1
Unsaturated C_{20-22}	—	—	—	—	1·4	1·2

The small quantity of fraction B necessitated the computation of its component glycerides directly from the component acid proportions but, in view of its I.V., it is most unlikely that it contained either fully saturated or tri-unsaturated glycerides.

The fully saturated glycerides (and their component acids) were determined in the case of fraction A with the results shown in Table 5, the crude product being separated into two portions by crystallization from ether.

The terms 'oleo'-, 'oleic', 'olein', denote the inclusion of octadecadienoic with oleic compounds.

Table 5. *Fully saturated glycerides present in fraction A of the external tissue fat*

	A			
Wt. of fraction oxidized (g.)	100·1			
	(i)		(ii)	
Wt. of crude F.S.G. (g.)	13·55		4·64	
I.V. of crude F.S.G.	0·3		0·8	
Sap. equiv. of crude F.S.G.	282·1		277·7	
Acid value of crude F.S.G.	1·7		1·8	
% (wt.)	13·1		4·4	
% (mol.)	13·2		4·5	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Component acids:				
Myristic	5·9	7·0	—	—
Palmitic	36·4	38·3	69·9	72·0
Stearic	57·4	54·5	30·1	28·0
Arachidic	0·3	0·2	—	—

A portion (89 g.) of the most soluble fraction C, after complete hydrogenation, gave the following fractions when crystallized from anhydrous ether:

	Least soluble				Most soluble
Wt. (g.)	25·1	10·2	11·1	5·4	37·3
I.V.	0·0	0·0	0·5	2·1	2·3
Sap. equiv.	290·0	287·7	285·3	282·4	286·0
% (wt.)	28·2	11·5	12·4	6·0	41·9
% (mol.)	28·0	11·4	12·5	6·1	42·0

The component acids in the least soluble portion were found (by ester-fractionation) to be palmitic 25·1, stearic 73·8, and C_{20-22} 1·1% (mol.), corresponding with an estimated proportion of 24·8% (mol.) of tri-C_{18} glycerides (including the C_{20-22} group), or 6·9% (mol.) in the whole of fraction C. The slight unsaturation in the more soluble fractions, in terms of oleodistearin, corresponded to 3·9% (mol.) of fraction C. The total tri-C_{18} glycerides in fraction C were thus estimated to form 10·8% (mol.) of this fraction, or 6·9% (mol.) of the total external tissue fat.

Consideration of the above data led to a final estimate of the glycerides probably present in each fraction A, B and C, including limiting values in certain cases (Table 6).

Discussion

If the molar percentage proportions of the component acids in the whole fats, determined by summation of the data from the three fractions into which the fats were separated by crystallization from acetone (Tables 3 and 6), are compared with those obtained from the ester-fractionation analyses of the total fatty acids from each fat (p. 941), it will be seen that very close accordance is shown by the figures for the perinephric fat, and somewhat less close, but on the whole satisfactory, agreement in the case of the external tissue fat. In both fats, however, resolution into several fractions has, as frequently happens, led to the appearance of minor component acids (lauric, arachidic), which were not detected in the analyses of the total fatty acids, and to apparent increases in the proportions of other minor component acids (hexadecenoic, octadecadienoic). Inspection of Tables 1 and 4 shows, moreover, that the minor component octadecadienoic acid is concentrated in the most soluble (mainly diunsaturated) glyceride fractions, whilst arachidic acid is mainly found in the least soluble (largely disaturated) fractions. This is merely a consequence of the circumstance

Table 6. *Probable component glycerides (% mol.) present in the constituent fractions and in the whole ewe external tissue fat*

	A		B	C	Whole fat
Glycerides	31·0		5·0	64·0	100·0
Component acids (increments):					
Myristic	1·0		0·1	2·7	3·8
Palmitic	10·2		1·6	19·7	31·5
Stearic	9·1		0·8	4·6	14·5
Arachidic	0·1		—	0·3	0·4
Tetradecenoic	Trace		—	0·2	0·2
Hexadecenoic	0·3		0·2	0·5	1·0
Oleic	10·3		2·2	33·3	45·8
Octadecadienoic	—		0·1	2·0	2·1
Unsaturated C_{20-22}	—		—	0·7	0·7
	F.S.G. (5·5%)	Mixed (25·5%)			
Groups of component acids (increments):					
C_{16} (+C_{14})	2·9	8·6	1·9	23·2	36·6
Stearic (+C_{20})	2·6	6·6	0·8	4·8	14·8
'Oleic' (+C_{20-22})	—	10·3	2·3	36·0	48·6
Groups of component glycerides (increments):					
Tripalmitin	0·2	—	—	—	0·2
Dipalmitomono-C_{18}	2·7	—	0·1	12·4	15·2
Palmitodi-C_{18}	2·6	24·9	4·9	44·7	77·1
Tri-C_{18}	—	0·6	—	6·9	7·5
Fully saturated	5·5	—	—	—	5·5
Mono-'oleo'-disaturated	—	19·3	2·5	20·1–27·0	41·9–48·8
Di-'oleo'-monosaturated	—	6·2	2·5	43·9–30·1	52·6–38·8
Tri-'olein'	—	—	—	Nil–6·9	Nil–6·9
	Probable component glycerides				
Fully saturated (5·5%):					
Tripalmitin	0·2	—	—	—	0·2
Dipalmitostearin	2·7	—	—	—	2·7
Palmitodistearin	2·6	—	—	—	2·6
Mono-'oleo'-disaturated (41·9–48·9%):					
Hexadecenopalmitostearin	—	0·9	0·5	—	1·4
'Oleo'-dipalmitin	—	—	0·1	12·4	12·5
'Oleo'-palmitostearin	—	17·8	1·9	7·7–14·6	27·4–34·4
'Oleo'-distearin	—	0·6	—	—	0·6
Di-'oleo'-monosaturated (52·6–38·7%):					
Palmitodi-'olein'	—	6·2	2·5	37·0–30·1	45·7–38·7
Stearodi-'olein'	—	—	—	6·9–Nil	6·9–Nil
Tri-'olein' (Nil–6·9%)	—	—	—	Nil–6·9	Nil–6·9

that, whilst nearly all the triglyceride molecules contain at least one radical of the most abundant acids oleic and palmitic, the minor component acids will only contribute one acyl group to any triglyceride molecule in which they are present. Thus the most likely combinations of the respective acids would be octadecadieno-oleo-saturated or arachido-palmito-unsaturated glycerides, of respectively large and small relative solubility in acetone.

The general glyceride structure of the two ewe fats conforms with that of other animal depot fats rich in stearic acid. This is indicated in the first place by the proportions of fully saturated glycerides which, when plotted against the respective proportions of saturated acids in the total fatty acids, fall on the

curve for this relationship characteristic for animal depot and milk fats [Hilditch, 1940, pp. 233, 237]. The more detailed examination of each fat shows that the chief component glycerides present are 'oleo'-palmitostearins and palmitodi-'oleins', which together form about 65% of the perinephric fat and nearly 75% of the external tissue fat. In the more saturated perinephric fat (the greater saturation being due to greater proportions of combined stearic acid) 'oleo'-palmitostearins occur to the extent of about 40%, whilst in the external tissue fat (with less stearic and more oleic acid in combination) these are reduced to about 30%, and over 40% of palmitodi-'oleins' are present. These facts, together with the presence of 10% of palmitodistearin in the perinephric fat, support the view that the stearo-glycerides of these and similar animal depot fats have been produced by hydrogenation of preformed oleo-glycerides.

It has already been explained that the experimental evidence, available from study of the group of mainly unsaturated glycerides most soluble in acetone, does not permit a decision as to whether the tri-C_{18} glycerides therein are stearodi-'oleins' or tri-'oleins', and therefore the results are given in terms of limiting values for these glycerides (Tables 3 and 6). From the general results obtained over a wide range of animal and vegetable fats in this laboratory, it appears increasingly doubtful whether, in instances such as the ewe fats now discussed, tri-unsaturated glycerides are present. At all events it is very unlikely that their proportions approach the upper limits given. For comparative purposes the presence of tri-'oleins' in the ewe fats may probably be safely ignored, and the approximate proportions of the component glycerides accepted as shown below (Table 7).

Table 7. *Component glycerides of ewe perinephric and external tissue fats* (% *mol.*)

	Perinephric	External tissue
% Stearic acid	25·6	14·5
Fully saturated:		
Tripalmitin	0·4	0·2
Dipalmitostearin	3·5	2·7
Palmitodistearin	10·2	2·6
Tristearin	0·3	—
Mono-'oleo'-disaturated:		
Hexadecenopalmitostearin	1·5	1·4
'Oleo'-dipalmitin	5·1	12·5
'Oleo'-palmitostearin	39·3	27·5
'Oleo'-distearin	1·4	0·6
Di-'oleo'-monosaturated:		
Palmitodi-'olein'	25·2	45·6
Stearodi-'olein'	13·1	6·9

If, after allowing for the composition of fully saturated glycerides present in the fats, the 'oleic' acid present is divided in proportion to the palmitic and stearic acids present in the remaining mixed ('oleo'-saturated) glycerides, and combined arithmetically with these two acids, a 'calculated' composition for the four mixed glycerides 'oleo'-dipalmitin, 'oleo'-palmitostearin, palmitodi-'olein' and stearodi-'olein' is derived which represents the maximum 'even distribution' of these acids in the form of mixed glycerides. The accordance between these 'calculated' figures and the proportions actually observed was remarkable in the cases of ox depot fats, English [Hilditch & Paul, 1938] and Indian [Hilditch & Murti, 1940], but in two pig depot fats [Hilditch & Pedelty, 1940] there was little resemblance between the two sets of values. In the present

instances there is again considerable accordance, although the respective figures are not so similar as in the ox depot fats mentioned:

Ewe depot fats	Perinephric		External tissue	
	Found	'Calc.'	Found	'Calc.'
'Oleo'-dipalmitin	5	8	13	20
'Oleo'-palmitostearin	41	41	28	23
Palmitodi-'olein'	25	21	46	38
Stearodi-'olein'	13	16	7	14

The implication of the frequent resemblance between the observed and 'calculated' proportions of the four minor classes of animal depot fat glycerides is somewhat uncertain, but there now seems little doubt that the accordances hold fairly closely in the more saturated fats of the group (i.e. those in which the saturated acids form about 50% or more of the total acids, and in which the stearic acid content approaches or exceeds about 20%). Of the three ox, two sheep, and two pig fats which have now been examined, these coincidences occur in all but the pig fats (the least saturated of the series). The following explanation of these observations may, however, be put forward. It has been shown that the proportions of these four groups of glycerides can be similarly calculated from the proportions of palmitic, stearic and oleic acids in certain vegetable seed fats, in which these acids are all major components, and which follow the 'rule of even distribution' very closely [Hilditch & Stainsby, 1936; Hilditch & Ichaporia, 1938]; these fats, however, contain only minor quantities of fully saturated glycerides. If a similar 'evenly distributed' mixture of palmito-oleo glycerides and triolein (such as is believed to be the precursor of the stearic-rich animal depot fats) undergoes partial hydrogenation before deposition as reserve fat, the conversion of oleic acid into stearic groups may well follow a similar random course, with the result that, when a sufficient proportion of oleo-glycerides have been thus transformed, the composition of the mixture can be approximately simulated by an arithmetical calculation based on division of the oleic acid still present in proportion to the palmitic and stearic acid contents of the animal depot fats.

Comparison of the chief component glycerides of pig, sheep and ox depot fats

The present work has completed a series of studies in which seven fats from the three species mentioned have been investigated by the acetone-crystallization procedure, leading to more detailed knowledge of the proportions of their various component glycerides than was hitherto available. It is therefore possible to review the series as a whole, with special reference to the manner in which the proportions of the minor groups of mixed glycerides are influenced by the proportions of oleic, stearic and palmitic acids present in the whole fats. For this purpose the data may be arranged in the form shown in Table 8.

In Table 8, the glyceride figures are based on the minimum limits of tri-'oleins' present (cf. p. 947); the small proportions of hexadecenopalmitostearins observed are included with the 'oleo'-palmitostearins; and the data are arranged in roughly decreasing order of general unsaturation. The palmitic acid content of the Bombay cow fat, and also the combined palmitic and myristic acid contents of the Calicut cow fat, are abnormally high, and this to some extent complicates comparison as regards the development of mixed stearoglycerides with increasing general saturation in the fats. Nevertheless, it is instructive to

Table 8. *Component glycerides of pig, sheep and ox depot fats* (% *mol.*)

Animal	Ewe	Pig	Pig	Ewe	Ox	Cow	Cow
Fat	External	Back	Peri-nephric	Peri-nephric	English	Calicut	Bombay
	(Present work)	[Hilditch & Pedelty, 1940]		(Present work)	[Hilditch & Paul, 1938]	[Hilditch & Murti, 1940]	
Component acids:							
Myristic (+C_{12})	3·8	1·3	1·8	3·0	2·6	5·7	5·9
Palmitic	31·5	29·0	31·1	27·1	33·4	33·4	40·8
Stearic	14·5	13·8	17·6	25·6	21·4	27·9	25·5
Arachidic	0·4	—	—	1·5	1·3	0·5	0·7
Hexa-(+tetra-)decenoic	1·2	2·7	2·4	2·0	2·5	1·9	2·8
Oleic	45·8	43·9	40·6	37·1	35·2	29·0	22·9
Octadecadienoic	2·1	7·2	5·3	3·3	3·5	1·5	1·1
Unsaturated C_{20-22}	0·7	2·1	1·2	0·4	0·1	0·1	0·3
Component glycerides (approx.):							
Fully saturated:							
Tripalmitin	Trace	1	—	Trace	3	—	3
Dipalmitostearin	3	2	4	4	8	16	23
Palmitodistearin	2	2	5	10	6	12	10
Tristearin	—	—	—	Trace	—	—	—
Mono-'oleo'-disaturated:							
'Oleo'-dipalmitin	13	5	9	5	15	11	18
'Oleo'-palmitostearin	28	27	34	41	32	38	34
'Oleo'-distearin	1	—	—	2	2	2	—
Di-'oleo'-monosaturated:							
Palmitodi-'olein'	46	53	40	25	23	17	11
Stearodi-'olein'	7	7	5	13	11	3	1
Tri-'oleins'	0	3	3	0	0	0	0

compare the relative proportions of palmitodi-'oleins', 'oleo'-palmitostearins and palmitodistearins as the proportion of stearic acid in the whole fat increases (Table 9).

Table 9. *Relative proportions of palmitodi-'oleins', 'oleo'-palmitostearins and palmitodistearins*

Animal ...	Pig	Ewe	Pig	Ox	Ewe	Cow	Cow
Fat ...	Back	External	Peri-nephric	English	Peri-nephric	Calicut	Bombay
C_{18} fatty acids:							
Stearic	13·8	14·5	17·6	21·4	25·6	27·9	25·5
Oleic + octadecadienoic	51·1	47·9	45·9	38·7	40·4	30·5	24·0
Palmitodi-C_{18} glycerides (increments %):							
Palmitodi-'oleins'	53	46	40	23	25	17	11
'Oleo'-palmitostearins	27	28	34	32	41	38	34
Palmitodistearins	2	2	5	6	10	12	10
	82	76	79	61	76	67	55
Palmitodi-C_{18} glycerides (%):							
Palmitodi-'oleins'	65	61	51	38	33	25	20
'Oleo'-palmitostearins	33	37	43	52	54	57	62
Palmitodistearins	2	2	6	10	13	18	18

The progressive change in the relative proportions of the three types of palmitodi-C_{18} glycerides, shown at the base of Table 9, is an extremely strong argument in favour of the hydrogenation hypothesis originally put forward by Banks & Hilditch [1931; 1932] to account for the varying proportions of fully saturated glycerides in these animal depot fats.

A somewhat similar comparison may be made in regard to the relative proportions of 'oleo'-dipalmitin and dipalmitostearin in the dipalmitomono-C_{18} group of glycerides. Here, however, the total proportion of the dipalmito-glycerides is partly dependent upon the palmitic acid content in the whole fats; the latter rises considerably above the average in the Indian cow depot fats with consequent increase in the amount of dipalmito-glycerides at the expense of the monopalmitodi-C_{18} group. Apart from increased proportions of dipalmito-glycerides from this cause, however, a tendency has previously been observed [Hilditch & Pedelty, 1940] for this group to increase in amount with increasing general saturation in the fats as a whole. The relevant data on this aspect for the seven fats for which information is now available are summarized in Table 10; in this case, since the minor quantities of lower saturated acids present are perforce included with palmitic acid in the evaluation of the main component glycerides, comparison is made with the combined amount of palmitic, myristic and lauric acids in the total acids of the fats.

Table 10. *Relative proportions of 'oleo'-dipalmitin and dipalmitostearin*

Animal ...	Pig	Ewe	Pig	Ox	Ewe	Cow	Cow
Fat ...	Back	External	Peri-nephric	English	Peri-nephric	Calicut	Bombay
Fatty acids:							
Palmitic (+C_{14}, C_{12})	30·3	35·3	32·9	36·0	30·1	39·1	46·7
Stearic	13·8	14·5	17·6	21·4	25·6	27·9	25·5
Total unsaturated	55·9	49·8	49·5	41·3	42·8	32·5	27·1
Dipalmitomono-C_{18} glycerides (increments %):							
'Oleo'-dipalmitins	5	13	9	15	5	11	18
Dipalmitostearins	2	3	4	8	4	16	23
	7	16	13	23	9	27	41
Dipalmitomono-C_{18} glycerides (%):							
'Oleo'-dipalmitins	71	81	69	65	56	41	44
Dipalmitostearins	29	19	31	35	44	59	56

The total amount of dipalmito-glycerides in the depot fats bears a general, though somewhat irregular, relationship to the proportion of palmitic acid present in the whole fat; but the relative proportions of 'oleo'- and stearo-dipalmitins are, as in the monopalmito-di-C_{18}-glycerides, governed entirely by the proportion of stearic and unsaturated acids present and thus again support the view that production of the more saturated component glycerides involves hydrogenation of preformed 'oleo'-glycerides—in this case 'oleo'-dipalmitins.

Summary

Specimens of perinephric and external tissue fats from the same ewe have been studied after resolution into fractions of varying degrees of solubility in acetone, and the approximate proportions of each of the major component mixed glycerides have been deduced from the resulting analyses (cf. Table 7). Sheep depot fats resemble ox and pig depot fats in that they are mainly composed of palmitodi-'oleins' and 'oleo'-palmitostearins, together with 'oleo'-dipalmitins, stearodi-'oleins' and minor amounts of other mixed glycerides. Triolein is either absent, or present in very small proportions.

The detailed data now available for the component glycerides of three ox, two pig and two sheep depot fats (the pig and sheep fats comprising perinephric

and external tissue fats from the same animal) have been reviewed, with special reference to the respective proportions of palmitodi-'oleins', 'oleo'-palmito-stearins and palmito-distearins, and of 'oleo'-dipalmitins and dipalmito-stearins, in relation to (*a*) the general unsaturation and (*b*) the palmitic acid contents of each fat as a whole.

The present study is the conclusion of a programme of work undertaken on behalf of the Food Investigation Board, and is published by permission of the Department of Scientific and Industrial Research.

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105. THE DETERMINATION OF SULPHANILAMIDE AND ITS DERIVATIVES

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THE extensive use of sulphanilamide and its derivatives in medicine during the past few years has naturally led to the investigation of methods for the estimation of this group of drugs in blood, especially as it seems that a knowledge of the blood level of the compound used is necessary for correct treatment. The work of Marshall *et al.* [1937] has shown that sulphanilamide is acetylated in the animal organism, and, since the therapeutic value is very considerably altered by this conjugation, a knowledge of the amount of this bound sulphanilamide as well as of the free compound is necessary.

The methods for the estimation of sulphanilamide in blood may be divided into two groups. In the first of these the sulphanilamide is diazotized, and the diazonium salt coupled with a suitable aromatic amine or phenol to give an intensely coloured azo dyestuff which may be determined colorimetrically. The coupling may be carried out in alkaline solution [Fuller, 1937], or in acid solution [Marshall, 1937–8; Scudi, 1937–8; Bratton & Marshall, 1939]. In the second group the sulphanilamide is combined directly with a suitable compound to give a coloured complex which is determined colorimetrically. Examples of this latter group are the sodium β-naphthaquinone-4-sulphonate method [Schmidt, 1937–8], and the *p*-dimethylaminobenzaldehyde methods described later.

The diazotization methods, especially that of Bratton & Marshall [1939], are extremely sensitive, and have been widely used. They have, however, several disadvantages, such as impermanence of colour, the use of unstable reagents, and interference by other substances such as traces of metals [Marshall, 1937–8], thiocyanates [Gregerson & Painter, 1938] etc.

Quantitative methods, based on the yellow colour given by sulphanilamide with *p*-dimethylaminobenzaldehyde in acid solution, have been worked out by Kühnau [1938], Kimmig [1938] and Werner [1939], while the latter's work has been partly substantiated by Andrews & Strauss [1941]. The advantages of the method as summarized by Andrews & Strauss are simplicity and rapidity of manipulation, and permanence of colour.

Work in this laboratory required a method for the determination of sulphanilamide in blood which would give accurate results in the presence of relatively high concentrations of thiocyanate, and accordingly Werner's [1939] method was studied. While, in agreement with Andrews & Strauss [1941], reasonably good results were obtained for free sulphanilamide, the values for acetylsulphanilamide were so erratic as to be worthless.

Closer study has revealed the following sources of error in Werner's [1939] method.

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(1) No provision is made for laking the blood before precipitation of the blood proteins. Marshall [1937–8] has indicated that this leads to low results, presumably due to incomplete equilibrium between the sulphanilamide in plasma and erythrocytes.

(2) The precipitation of the blood proteins is carried out at an insufficiently great dilution. Marshall [1937–8] has shown that while recovery of added sulphanilamide from blood is reasonably complete after precipitation in 1 : 10 dilution, recovery of added acetylsulphanilamide is not quantitative even in 1 : 20 dilution. Werner has used 1 : 5 dilutions, although his own results show that recovery of acetylsulphanilamide is incomplete in 1 : 10 dilutions under the conditions used by him. Work in this laboratory described later indicates that quantitative recovery of acetylsulphanilamide from blood may be obtained only in 1 : 20 dilutions.

(3) The use of trichloroacetic acid as hydrolysing agent for acetylsulphanilamide is unsatisfactory. Marshall [1937–8] has suggested that this is due to the decomposition of the trichloroacetic acid during the heating necessary for complete hydrolysis of the acetylsulphanilamide. This is borne out by experiments in this laboratory, which have shown that a 4% solution of trichloroacetic acid as used by Werner is approximately 50% decomposed by 30 min. heating at 100°. The extent of decomposition varies with the conditions of heating, such as shape of tubes used etc., and is not exactly reproducible. Apart from changes in the rate of hydrolysis, the variations in acid concentration will lead to corresponding variations in *p*H after partial neutralization, and as will be seen later the *p*H is of critical importance for reproducibility of colour.

(4) The *p*H of the solution in which the colour is developed is only partially controlled. Evidence is presented in the experimental section which shows that there is an optimal *p*H zone within which alone reproducible results can be obtained. Outside the limits of this zone a very marked decrease in colour intensity is observed. Werner [1939] observed that the presence of free trichloroacetic acid interfered with the maximal production of colour. The work of Kühnau [1938] has shown that this effect is not a specific property of trichloroacetic acid, since he estimated uliron (N^4-sulphanilyl-N^1N^1-dimethylsulphanilamide) in blood by addition of a trichloroacetic acid solution of *p*-dimethylaminobenzaldehyde to a trichloroacetic acid blood filtrate without previous neutralization of the blood filtrate. Work in this laboratory has shown that the effect can be observed with a variety of acids including hydrochloric, sulphuric, *p*-toluenesulphonic and salicylsulphonic acids. The effect thus appears to be entirely a matter of *p*H. Werner attempted to overcome the difficulty by partial neutralization of the trichloroacetic acid filtrate with NaOH. The system trichloroacetic acid-sodium trichloroacetate is, however, a comparatively badly poised buffer since trichloroacetic acid is a relatively strong acid, and small pipetting errors might lead to a large shift in *p*H. A much greater source of error is the decomposition of trichloroacetic acid during heating in the determination of acetylsulphanilamide, and as will be seen from the last section, this is very difficult to control.

(5) The amount of *p*-dimethylaminobenzaldehyde used is insufficient for maximal colour development. While it is true that by the addition of exactly the same amount of *p*-dimethylaminobenzaldehyde reagent in every case, reproducible results will be obtained, such reproducibility will obviously depend on the accuracy of measurement of the reagent, and on the stability of the reagent solution. This is evidently undesirable for a routine clinical method. It will be seen from Fig. 2 (experimental section), that there is an optimal zone within

which small variations in the amount of *p*-dimethylaminobenzaldehyde reagent added produce practically no difference in colour.

The sources of error recapitulated above have been avoided in the method to be described by the following precautions.

(1) Complete laking of the blood before precipitation of proteins.

(2) Protein precipitation in 1 : 20 dilution.

(3) The use of a *p*-toluenesulphonic acid-HCl mixture for precipitation of the blood proteins, and subsequent hydrolysis of the acetylsulphanilamide. This mixture is unaffected by much longer heating than is necessary to effect quantitative hydrolysis.

(4) The use of a citrate-HCl buffer to maintain the *p*H within the optimal zone.

(5) The amount of *p*-dimethylaminobenzaldehyde is regulated to fall within the optimal zone.

Experimental

The determination of sulphanilamide in blood

Reagents.

(1) 20 g. *p*-toluenesulphonic acid in 100 ml. 0·2*N* HCl.

(2) 0·75*M* disodium hydrogen citrate solution. 39·4 g. 'Analar' citric acid are dissolved in 188 ml. 2*N* NaOH, and diluted to 250 ml.

(3) 2% alcoholic *p*-dimethylaminobenzaldehyde solution. 2·00 g. 'Analar' *p*-dimethylaminobenzaldehyde are dissolved in 100 ml. 95% alcohol. In estimations with the Pulfrich photometer or similar instruments in which the colour of the blank is compensated, the purity of the *p*-dimethylaminobenzaldehyde is not of very great importance since identical values were given by a 3 years old yellow specimen, and by a colourless preparation obtained by the method of Adams & Coleman [1922]. In estimations with the ordinary colorimeter, however, as Andrews & Strauss [1941] have noted, the question of purity is of importance, since certain preparations have a greenish colour which leads to high blank values. Such specimens can be purified by fractional precipitation from acid solution by the method of Adams & Coleman [1922].

All the above reagents appear to be stable indefinitely at 0°.

Method

1·0 ml. of whole blood is measured into 13·0 ml. of distilled water, the mixture shaken vigorously, and then kept for about 3 min., when laking is complete. 6·0 ml. of the 20% *p*-toluenesulphonic acid reagent are added slowly with vigorous shaking. The mixture is kept at least 5 min., and filtered through a Whatman No. 40 filter paper.

For the determination of free sulphanilamide, 5 ml. of the filtrate are treated with 1 ml. of the disodium hydrogen citrate solution, and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution. The yellow colour develops immediately to its maximum value, and is stable for at least a week, although if specimens are to be kept for more than 12 hr. it is advisable to stopper the tubes, as otherwise evaporation of the alcohol may lead to separation of crystals of *p*-dimethylaminobenzaldehyde.

For the determination of total sulphanilamide (free and acetylsulphanilamide), 5 ml. of the filtrate are transferred to a graduated tube, and heated for 60 min. in a boiling water bath. The tube is then cooled, the contents diluted to 5 ml. with water, and the colour developed as in the determination of free sulphanilamide.

Blanks for photometric determinations are made by mixing 1·5 ml. of the *p*-toluenesulphonic acid reagent, 1 ml. of the citrate reagent and 2 ml. of the *p*-dimethylaminobenzaldehyde reagent, and diluting to 8 ml. with water.

It must be emphasized that the coloured solutions may not be diluted, since the alteration in the ionic strength of the buffer apparently produces a change of *p*H, and a marked relative decrease in colour is observed.

Measurement of colour

The majority of the determinations have been carried out with the Pulfrich photometer using filter S 47, and 5 mm. and 10 mm. cells according to the intensity of colour. Then

$$\text{mg. sulphanilamide/100 ml. blood} = E \times 11{\cdot}7,$$

where E is the extinction for 10 mm. solution thickness using filter S 47. The value of the constant should of course be determined independently in the user's laboratory, since small variations may occur, owing to variations in filters or reagents.

Some determinations have also been made with the Hilger Spekker photoelectric absorptiometer. In this case the most suitable filter is the Ilford Spectrum Blue, which has maximum transmission at 4700 Å.

Since Beer's law is valid for the concentration range studied (0·5–12 mg. sulphanilamide/100 ml. blood), the method should be applicable to the ordinary colorimeter, provided that *p*-dimethylaminobenzaldehyde of adequate purity is available. The difficulty of matching yellow solutions can be obviated by the use of a suitable blue filter. The question of suitable standard solutions is discussed in the paper of Andrews & Strauss [1941]. The use of permanent standards prepared from potassium chromate solutions as suggested by Werner [1939] is not recommended for accurate work, as such solutions have a different absorption spectrum from the solutions to be measured.

Results

Recoveries of sulphanilamide and acetylsulphanilamide from blood in 1:10 and 1:20 dilutions are shown below.

Table 1

Dilution 1 : 10

Substance	Added mg./100 ml.	Found mg./100 ml.	% recovery
Sulphanilamide	3·2	3·2	100
	6·4	6·35	99
	9·6	9·45	98
Acetylsulphanilamide	4·0	4·0	100
	8·0	7·85	98
	12·0	11·2	93

Table 2

Dilution 1 : 20

Substance	Added mg./100 ml.	Found mg./100 ml.	% recovery
Acetylsulphanilamide	4·0	4·0	100
	8·0	8·0	100
	12·0	11·9	99

Each of the above values is the mean of three estimations.

Derivatives of sulphanilamide

In addition to sulphanilamide itself, three derivatives in common use in medicine have been studied, albucid (*p*-aminobenzenesulphonacetamide), sulphapyridine (dagenan, M & B 693, 2-sulphanilamidopyridine) and sulphathiazole (2-sulphanilamidothiazole). All three compounds give yellow colours with *p*-dimethylaminobenzaldehyde in acid solution, which may be used for their estimation. The method used is identical with that used for sulphanilamide. The molecular extinction coefficients for sulphanilamide, sulphapyridine and sulphathiazole are identical, so that the latter substances may be estimated by applying a molecular weight correction to the sulphanilamide factor. Thus:

$$\text{mg. sulphapyridine/100 ml. blood} = E_1 \times 16{\cdot}0,$$

and

$$\text{mg. sulphathiazole/100 ml. blood} = E_2 \times 17{\cdot}35,$$

where E_1 and E_2 are the respective extinctions for 10 mm. solution thickness using filter S 47 of the Pulfrich photometer.

In cases where low blood levels of sulphapyridine and sulphathiazole are anticipated, 20 mm. cells should be used with the Pulfrich photometer.

In the case of albucid, the theoretical extinction coefficient is not obtained unless the albucid solution is heated for 60 min. at 100° as in the case of the acetylsulphanilamide estimation. In direct determinations slightly low but consistent values are obtained, possibly owing to the presence of *p*-acetylaminobenzenesulphonacetamide in the preparation used. In such cases a separate calibration curve must be made.

The *p*-dimethylaminobenzaldehyde method appears to be applicable to all derivatives of sulphanilamide which have a free primary amino group directly attached to an aromatic residue. Its range of utility is thus similar to that of the diazotization methods.

The variation of colour with hydrogen ion concentration

Buffer solutions were made up with varying ratios of 0·75*M* disodium hydrogen citrate and 0·75*N* HCl. The *p*H values of these were determined

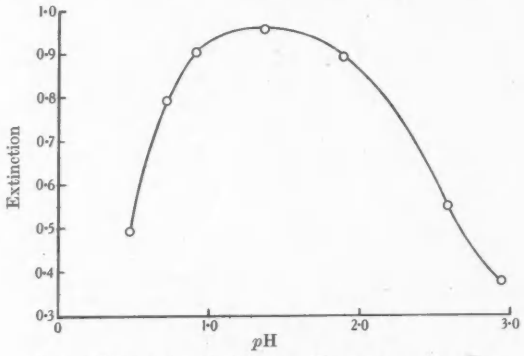

Fig. 1. The relationship between *p*H and colour intensity.

electrometrically using the quinhydrone electrode. 5 ml. portions of the buffers were treated with 0·30 ml. of a sulphanilamide solution containing 8·0 mg./100 ml., and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution added. The results are shown in Fig. 1.

The variation of colour with p-*dimethylaminobenzaldehyde concentration*

A series of tubes were set up containing 0·3 ml. 8 mg./100 ml. sulphanilamide solution, 1·5 ml. *p*-toluenesulphonic acid reagent, 1 ml. disodium hydrogen citrate reagent and 3·2 ml. of water. Varying volumes of the 2% alcoholic *p*-dimethylaminobenzaldehyde reagent were added, and the contents of the

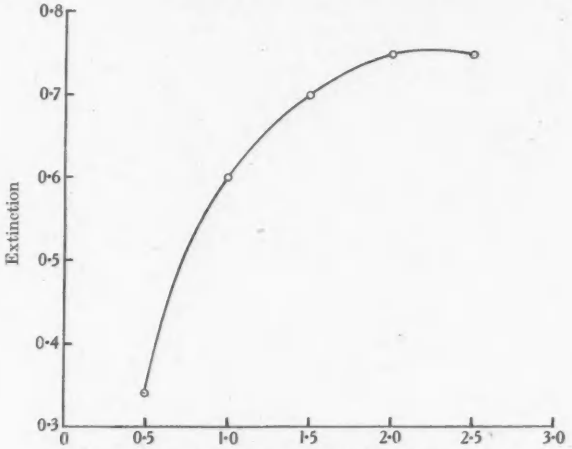

Fig. 2. The relationship between *p*-dimethylaminobenzaldehyde concentration and colour intensity.

tubes were diluted to 8·5 ml. with 95% alcohol. A series of corresponding blanks were set up in which the 0·3 ml. of sulphanilamide was replaced by 0·3 ml. of water. Each tube was measured against the corresponding blank. The results are shown in Fig. 2.

Identification of the chromophoric complex as p-*dimethylaminobenzylidene*-p-*aminobenzenesulphonamide*

0·3 ml. of a solution of *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide [Gray *et al.* 1937] containing 14·10 mg. in 100 ml. *p*-toluenesulphonic acid reagent, was treated with 1·2 ml. of the *p*-toluenesulphonic acid reagent, 3·5 ml. of water, 1 ml. of the disodium hydrogen citrate reagent and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution. The extinction was measured using a 10 mm. cell and filter S 47.

0·3 ml. of an equivalent solution of sulphanilamide (8 mg./100 ml.) was treated with 1·5 ml. of the *p*-toluenesulphonic acid reagent, 3·2 ml. of water, 1 ml. of the disodium hydrogen citrate reagent and 2 ml. of the 2% alcoholic *p*-dimethylaminobenzaldehyde solution. The extinction was measured as above.

Table 3

Substance	Extinction
p-Dimethylaminobenzylidene-*p*-amino-benzenesulphonamide	0·885
Sulphanilamide	0·878

Discussion

Any explanation of the *p*H-colour, and *p*-dimethylaminobenzaldehyde concentration-colour relationships shown in Figs. 1 and 2 necessitates an examination of the reaction responsible for colour formation. The compound responsible for the colour produced when sulphanilamide reacts in acid solution with *p*-dimethylaminobenzaldehyde is probably the Schiff base *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide

$$(CH_3)_2{:}N.C_6H_4.CH{:}N.C_6H_4.SO_2.NH_2.$$

This base has been synthesized by Gray *et al.* [1937] by direct condensation of sulphanilamide with *p*-dimethylaminobenzaldehyde at 140°. Its melting point is given as 229°. Werner [1939], by condensation of sulphanilamide with *p*-dimethylaminobenzaldehyde in acid solution, prepared a substance of melting point 224°, for which he gives the anomalous formula $C_{15}H_{16}O_4N_3S$ (the Schiff base would be $C_{15}H_{17}O_2N_3S$). It is probable that the substance isolated by Werner was slightly impure *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide. It is shown in the experimental section that a preparation of this base synthesized according to the method of Gray *et al.* [1937] had, under appropriate conditions, the same colour intensity as that given by an equivalent amount of sulphanilamide in the colorimetric determination. It may therefore be concluded that the chromophoric complex is indeed *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide.

Dimroth & Zoeppnitz [1902], in the course of investigations into the mechanism of Schiff base formation, suggested the following reaction scheme:

$$\underset{\text{I}}{Ar.CHO} + NH_2Ar' \rightleftarrows \underset{\text{A}}{Ar.CHOH.NH.Ar'} \rightleftarrows \underset{\text{II}}{Ar.CH{:}N.Ar'} + \underset{\text{B}}{H_2O}.$$

They found that in those cases where the aromatic aldehyde had a *para*-substituted positive (electron-repelling) group, and the aromatic amine a *para*-substituted negative (electron-attracting) group, the amino-carbinol A was stable in strongly acid solution. Since these conditions are fulfilled in the reaction between sulphanilamide and *p*-dimethylaminobenzaldehyde, and since the amino-carbinol A would be expected to have a less intense colour than the Schiff base B, owing to the interruption of the series of conjugated double bonds, this may provide an explanation for the diminution in colour below *p*H 1 as shown in Fig. 1. The decreased colour above *p*H 2 may possibly be explained by the failure of reaction I to proceed to completion, or by the diminished ionization of the *p*-dimethylamino group.

The very large excess of *p*-dimethylaminobenzaldehyde necessary for maximum development of colour (Fig. 2) may also be explained by the reversible nature of the above mechanism.

Summary

1. The conditions affecting the reaction between sulphanilamide and *p*-dimethylaminobenzaldehyde in acid solution have been studied.

2. A simple and rapid colorimetric estimation of sulphanilamide and its derivatives in blood based on this reaction has been developed.

3. The compound responsible for the colour has been identified as *p*-dimethylaminobenzylidene-*p*-aminobenzenesulphonamide.

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106. THE ROLE OF THE PHOSPHOLIPIN IN VISUAL PURPLE SOLUTIONS

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For sixty years since its discovery by Boll [1876] and Kühne [1877–8] visual purple has been identified exclusively through the absorption spectrum and the photosensitivity of its solutions. Recently more properties of this chromoprotein have been studied, namely its diffusion coefficient [Hecht *et al.* 1937], molecular weight [Hecht & Pickels, 1938], chromophore carrier weight [Broda *et al.* 1940], cataphoretic mobility [Broda *et al.* 1939; Broda & Victor, 1940], and some properties of its chromophore [Wald, 1938]. All these determinations, except that of the cataphoretic mobility, depend, however, ultimately on a knowledge of light absorption. Up to the present the spectrum and the photosensitivity have been used as the only purity criteria of visual purple.

The determination of either the spectrum or the photosensitivity, however, even in the purest solutions is complicated by the presence of coloured (yellow) 'impurities' [Dartnall *et al.* 1936]. Obviously the term 'impurity' must not imply more than that the substance concerned is different from the protein carrier and from the chromophore of visual purple; its interaction with the chromoprotein may well be of physiological significance.

Lythgoe [1937] suggested that the impurities consist of lipins, while Krause [1937] proved the presence in visual purple solutions of phosphorus and choline, both constituents of lecithin. A phospholipin content of the solutions was indeed to be expected, since these lipins are contained in the retina and in other parts of the eye [cf. Krause, 1934, 1]. The outer limbs of the rods contain a phospholipin which is blackened by osmic acid; it has been called 'myeloid' by Kühne and is considered by Krause [1934, 2] to be lecithin. Schmidt [1938] assumes that there are alternating layers of protein and phospholipin in the outer limbs. Part of the phospholipins, although lipophil, will dissolve along with the chromoprotein during the preparation of the visual purple solution, their solubility in water being due to the formation of hybrid ions [Kuhn *et al.* 1935].

The object of the present work was to measure the concentration of the phospholipins, and to investigate whether they can account for the light absorption attributed to the 'yellow impurities'. This information should not only help to define visual purple more accurately, but also to interpret the sensitivity curve of the human eye.

The concentration of the phospholipin

When fresh rod tissue from dark-adapted frogs' eyes is treated with light petroleum before extraction of the visual purple with digitonin solution as described by Lythgoe [1937], a deep yellow extract is obtained. The dissolved substance contains only about 1 % P showing that in addition to phospholipins, sterols or fats are present. Neither sterols nor fat, however, can be taken up by aqueous digitonin, and therefore they cannot be present in the visual purple solution.

To determine the phospholipin concentration in a visual purple solution, a quantity of a pure solution (optical density at 500 mμ 1·20, at 400 mμ 0·37 cm.$^{-1}$) was bleached and evaporated to dryness in contact with $CaCO_3$ at room temperature. The residue was extracted with chloroform until practically free from P. The yellow extract was evaporated, the residue after incineration dissolved in HNO_3, and the P determined with molybdate. In this way the original solution was found to contain 0·0232 mg. P/ml. The fact that the whole of the phosphorus compound is soluble in chloroform as well as in water, justifies the calculation of the amount of phospholipin from the total P. If a P content of the lipin of 4% is assumed, the phospholipin concentration becomes 0·58 mg./ml. This corresponds to 0·48 mg./ml. for a solution diluted to standard optical density (1 cm.$^{-1}$ at 500 mμ).

The protein concentration in the standard solution is 1·2 mg./ml. [Broda *et al.* 1940]; hence the weight of the lipin present is comparable with that of the protein. It greatly exceeds that of the prosthetic group, as at least 17,000 g. of protein are required [Broda *et al.* 1940] to carry 1 mol. of the prosthetic group with a probable weight of the order of 500 g.

The spectrum of the retina phospholipin

The spectrum of the clear lipin solution obtained from rod tissue as described above was determined with a Hilger Spekker photometer (Table 1). For comparison, the spectrum of egg lecithin ('purissimum', Merck) was also taken. No

Table 1. *The light absorption of phospholipins*

Extinction coefficients, calc. on a g./litre basis; solutions in chloroform

	Wave-length in mμ					
	440	420	400	380	360	340
Retina phospholipin	0·035	0·045	0·06	0·10	0·18	0·51
Egg lecithin	0·035	0·04	0·045	0·055	0·07	0·15
β-Carotene [Zechmeister, 1934]	250	160	80	40	16	13

importance can be attached to numerical agreement or disagreement owing to the lack of purity of the retina phospholipin and the poor stability of phospholipins exposed to air and light. It is, however, found that the two substances have in common both the shape of the absorption curve and the order of magnitude of the extinction coefficient. This at 400 mμ is about 10^3 times smaller than that of the yellow pigments of the carotene or flavin classes.

In a qualitative sense the phospholipin accounts for the steady increase with decreasing wave-length of the absorption due to the impurities in the visible [Lythgoe, 1937] and ultraviolet [Goodeve, Lythgoe & Wood, unpublished]. Its presence explains why the ratio of the densities at 400 and at 500 mμ can be used as a purity criterion of visual purple [Lythgoe, 1937; Wald, 1938]. Finally, the oxidation of the phospholipins is probably the reason why the absorption of visual purple solutions over the whole range increases with time unless air is excluded.

To determine quantitatively the influence of the phospholipins on the absorption of the visual purple solutions, the absorption of an aqueous phospholipin solution with a concentration similar to that deduced from the P content of the visual purple solution would have to be measured. Unfortunately, however, aqueous phospholipin solutions free from protein are opalescent, although the solutions of visual purple like certain other protein solutions con-

taining phospholipin (e.g. solutions of euglobulin) are perfectly clear. Scattering must have greatly falsified the measurements of lecithin and kephalin from beef brain in water by Wadsworth & Crowe [1936], which are apparently the only determinations of a phospholipin spectrum so far carried out.

The optical density of the phospholipin in chloroform may, however, give an idea of the order of magnitude of its contribution to the total absorption of visual purple solutions. This way of approach appears to be valid, since the colour of phospholipin solutions is not greatly influenced by the solvent. A 1% solution of egg lecithin was tested and showed practically identical colours in amyl acetate, in carbon tetrachloride and in chloroform. The colours in hexane and in ethyl alcohol were slightly weaker, in benzene slightly stronger. The yellow colour of a solution in acetic acid had a faintly reddish tinge, while that of a solution in amyl alcohol had a faint greenish tinge. In no case, however, was the deviation of the colour from that in chloroform substantial. Above all, addition of water to the solutions did not produce any colour change as long as its amount was too small to make the solution opalescent.

On this basis, the contribution of the phospholipin to the density of a standard solution of visual purple would be about 0·02 at 440 mμ, 0·03 at 400 mμ, and 0·09 at 360 mμ. Clearly these values must not be taken to be more than rough approximations.

The influence of the phospholipin on the scotopic sensitivity curve of the eye

Schneider *et al.* [1939; 1941] found that the apparent quantum efficiency of the decomposition of visual purple in solution is nearly constant for wave-lengths greater than 440 mμ, but drops in the blue, violet and particularly in the ultraviolet. Its value at 400 mμ is only about 80%, and at 365 mμ only about 70% of that at 500 mμ. A genuine decrease of the quantum yield with decreasing wave-length would be unlikely. The apparent decrease was therefore attributed to the presence of an internal yellow filter in the solutions. This would absorb light without participating in the photoreaction, thus reducing the number of visual purple molecules decomposed per quantum absorbed by the solution as a whole. That is precisely how the phospholipins must act, since they absorb short wave light without contributing to the photoreaction. From the rough data for the extinction of the phospholipin, given in the preceding section, and taking into account that the density of the standard visual purple solution at 400 mμ is 0·3 and at 365 mμ 0·55 [Goodeve, Lythgoe & Wood, unpublished], it would be predicted that at 400 mμ about 10%, and at 360 mμ about 17% of the absorbed light would go to the phospholipin.

In the same spectral range there is a still greater discrepancy between the absorption curve of visual purple *in vitro* and the scotopic sensitivity curve of the human eye. As before, the two curves fit well above 500 mμ [Dartnall & Goodeve, 1937], but the ratio sensitivity : absorption at 365 mμ is only 1/4000 of its value at 500 mμ [Schneider *et al.* 1941]. This can hardly be due to any difference between the spectra of frog and human visual purples, which indeed has never been suggested. Schneider *et al.* [1941] pointed out that a yellow filter in the eye may be the reason of the disagreement. This filter may well consist of phospholipins. Much more lipin would be required to explain the discrepancy between the scotopic sensitivity curve and the absorption curve than to explain the drop in the apparent quantum efficiency in solution. This is not surprising, since only a small part of the retina lipin escapes the pre-extraction with light petroleum, and thus dissolves along with the chromoprotein.

Extrapolation from the extinction of the lipin in chloroform solution to 100% concentration shows that a lipin layer of a thickness of a fraction of a millimetre would impart to the filter in the eye the density 3·6 at 365 mμ, which is required by the results of Schneider *et al.* The lens and the vitreous humour contain some lipins, but their absorption is not very great [Graham, 1922]. It is concluded therefore that the bulk of the filter effect occurs in the retina. Although the light must travel over a greater distance in the lens and in the vitreous humour, the lipin concentration is much greater in the retina [Krause, 1934, 1]. It would be premature, however, to calculate the absorption in the retina as long as the extinction coefficient of the lipin *in situ* and the path of the light in the rods are not known.

The decrease with age of the sensitivity of the human eye to short-wave light will have to be correlated with the parallel increase of the lipin content of the eye tissues [cf. Krause, 1934, 1].

The bond between the visual purple and the phospholipin

The visual purple protein is possibly a globulin, since it is precipitated by removal of salts (dialysis) or by saturation of the neutral solution with sodium or magnesium sulphate [Kühne, 1877–8; Lythgoe, 1937]. Globulins interact specifically with phospholipins [Frazer, 1941]. Experiments with visual purple showed that the phospholipin cannot be removed from (bleached or unbleached) solutions by shaking with light petroleum; this points to chemical linkage of some kind as assumed for other lipoproteins on similar evidence by Mâchebœuf [1927]. The idea that the whole of the phospholipin is linked with the visual purple protein is supported by the clarity of the solutions; Sørensen [1930] has emphasized that the clarity of euglobulin solutions, which contain phospholipin, is due to a secondary bond. The fact that the d_{400}/d_{500} ratio of the purest solutions, as found in different laboratories, is nearly constant at about 0·3, indicates a roughly stoichiometric relation between the chromoprotein and the lipin, as far as the light-absorbing part of the lipin is concerned.

Further experiments showed that precipitated visual purple yields its phospholipin on treatment with solvents such as chloroform; electrodialysis also seems very effective in breaking the bond and removing the phosphorus. Only 2·5 mg. lipin were extractable from 18·3 mg. protein precipitated (and bleached) by 12 hr. electrodialysis through a collodion membrane at 220 v. Most of the phosphorus had been carried away through the membrane. The spectrum of the chloroform extract showed a maximum at 370 mμ indicating that it was due to indicator yellow (or 'retinene' in the terms of G. Wald) rather than to lipins. These facts suggest that the bond between the chromoprotein and the phospholipin is not strong.

Possibly visual purple solutions could be freed from lipin without bleaching or precipitating by electrodialysis in a flow of buffer.

SUMMARY

Solutions of visual purple contain phospholipin from the retina. Even in the purest solutions so far obtained the quantity of the lipin is comparable with that of the protein, and is much greater than that of the prosthetic group.

The light filter effect due to the phospholipin accounts, at least to a large extent, for the apparent decrease of the quantum efficiency of the decomposition of visual purple in solution in the blue, violet and particularly ultraviolet. The phospholipin in the retina serves to explain the discrepancy in the

same range between the photosensitivity curve of visual purple solutions and the human scotopic sensitivity curve.

The phospholipin is connected with the visual purple protein by a secondary bond which may be similar to that between euglobulin and lecithin.

Phospholipin is effectively removed from visual purple solutions by electrodialysis.

I am indebted to Dr C. F. Goodeve and the late Dr R. J. Lythgoe for enabling me to carry out this work and for their constant help, Miss S. J. Hooper for the preparation of the visual purple, and the Rockefeller Foundation for a financial grant.

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107. PHOTOELECTRIC ESTIMATION OF INDOLE

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INDOLE and its derivatives can be conveniently detected in small concentrations by means of the colours which they produce in the presence of acidified alcoholic *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). Unfortunately, these colours are often transient and do not lend themselves readily to quantitative work. This difficulty has been overcome by using a photoelectric colorimeter. With this instrument, a measurement of the optical density [log (incident intensity/transmitted intensity)] of a solution could be made within 7 sec. of the colour being developed, and the readings so obtained were reproducible. The method has been employed for concentrations up to an equivalent of 2 mg. of indole per 100 ml. of solution, by the technique about to be described.

Instrument

The colorimeter was a Hilger 'Absorptiometer' [Hilger, 1936]. 1 cm. cells were used with a combination of colour filters (Nos. 6 and 7) giving maximum transmission at about 4400 Å., this being found to give optimum sensitivity. The instrument scale is calibrated directly in optical densities.

Ehrlich's reagent

1·5 g. of *p*-dimethylaminobenzaldehyde were dissolved in 175 ml. of ethyl alcohol, and 30 ml. of conc. HCl were added [cf. Cole, 1933].

Method

5 ml. of the solution to be tested were diluted with an equal volume of water and 5 ml. of the reagent (which could be warmed previously with advantage) were added. The mixture was rapidly brought just to boiling point, when the colour was fully developed. The coloured solution was transferred immediately to a 1 cm. absorption cell and matched in the absorptiometer. With practice, the time from boiling to the completion of matching could be reduced to 7 sec. The density was then read, using water in the comparison cell. Since this last adjustment is made independently of the coloured solution, fading cannot affect the result once the initial matching is completed.

Calibration

A solution containing 1·6 mg. of pure indole (Roche Products: M.P. 52·5°) in 100 ml. of water was employed, and a series of standards containing 5, 4, 3, 2, 1 and 0 ml. of this in 10 ml. was prepared. Each was heated to boiling point in turn with 5 ml. of Ehrlich's reagent and its colour density measured. The colour produced by indole itself is quite stable and several settings of the absorptiometer scale could be made. At these low concentrations the colours are not intense, but there is a linear relationship between concentration and optical density which breaks down in stronger solutions. The values appeared to depend

on the age of the reagent and it was desirable to prepare a new calibration curve for each set of estimations. A typical calibration is set out in Table 1; the positive density at zero concentration of indole is due to the residual colour of the reagent.

Table 1. *Calibration of absorptiometer scale against indole concentrations*

Concentration of indole mg./100 ml.	Scale reading $d_{1\,cm.}$	Concentration of indole mg./100 ml.	Scale reading $d_{1\,cm.}$
1·60	0·185	0·64	0·090
1·28	0·152	0·32	0·059
0·96	0·122	0·00	0·029

Accuracy

Over this range of densities the scale is divided to 0·01 and can easily be read to 0·001 by interpolation. For 8 readings at 1·60 mg./100 ml. the observed variation gives an estimated probable error less than 0·001 in density, or less than 0·5 % in concentration, for the mean value; at 0·96 mg./100 ml. the error is about 0·8 %, and for the most dilute solution, where the probable error in density, over a series of readings, is still only 0·001, the probable error in concentration is 1·6 %.

SUMMARY

Small concentrations of indole and its derivatives can be estimated quickly and accurately with Ehrlich's *p*-dimethylaminobenzaldehyde reagent by the use of a photoelectric colorimeter.

This investigation was made in connexion with experiments for which the author received a personal grant from the Medical Research Council, to whom he expresses his thanks. He is also indebted to Adam Hilger Limited for the use of the absorptiometer.

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108. A GRAVIMETRIC METHOD FOR THE ESTIMATION OF BROMIDE IN ORGANIC MATERIAL[1]

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DURING the past decade interest in the problem of bromide intoxication has steadily increased and numerous methods of estimating bromide in biological fluids have been described. Three main types can be distinguished, colorimetric, volumetric and electrolytic. The first two are based on reactions of extreme delicacy which are not easily controlled. The last is not well suited to clinical investigation because large amounts of fluid as well as expensive and delicate apparatus are required. If information on the subject of bromism has not kept pace with the interest it has evoked, the inaccuracies of the present clinical methods can alone be blamed. The advantages of the method to be described are that it is based on a simple chemical reaction which for practical purposes is irreversible. The analysis cannot therefore be disturbed by slight departures from the standard conditions of the experiment, such as differences in colour of the materials to be analysed, temperature, concentration of the reagents and *p*H of the fluids. Moreover, the amounts of bromide and chloride in the material to be estimated are determined in one experiment.

In principle the method depends upon the difference between the weights of AgBr and AgCl. Both halides are precipitated at the outset with excess of a measured quantity of $AgNO_3$ and the precipitate is removed by filtration and its weight (w) is estimated. The excess of $AgNO_3$ is then precipitated with NaCl, and after filtration its weight (p) is determined. If c represents the weight of AgCl obtainable from the amount of standard solution of $AgNO_3$ taken and if x represents the amount of AgCl and y the amount of AgBr obtained from the unknown material, then

$$x+y=w, \tag{i}$$

$$x+\frac{143}{188}y+p=c, \tag{ii}$$

From (i) and (ii)

$$w-y=c-p-\frac{143}{188}y, \tag{iii}$$

and from (iii)

$$\frac{188}{45}(w+p-c)=y. \tag{iv}$$

But if y' represents the weight of NaBr in the unknown material, then

$$y'=\frac{103}{188}y, \tag{v}$$

and from (iv) and (v)

$$y'=\frac{103}{45}(w+p-c).$$

[1] Work undertaken on behalf of the Medical Research Council while Dr Brinton held a Halley Stewart Research Fellowship.

Special apparatus required

(1) Boiling tube of 50 mm. diameter and 260 ml. capacity. The neck of the boiling tube should be accurately ground to take a reflux condenser.

(2) Reflux condenser ground to fit boiling tube (1) and extraction tube (6).

(3) Rubber ring, round in section and of a size suitable for stretching over sintered glass filter (4).

(4) Two sintered glass filters (Schott und Gen., Jena), graded as having a porosity of 'less than 7' (No. 10 G 4). These are subsequently referred to in the text as 'filter-cups'. They are prepared in the manner described below.

(5) Special funnel into which filter-cups are placed for the purpose of filtration under pressure. The wide portion of the funnel is so shaped as to allow the free insertion of a filter-cup. The rubber ring stretched round the filter-cup forms an airtight joint between the funnel and the cup. The narrow lower portion of the funnel is inserted into a rubber stopper placed in a pressure flask of a suitable size. When negative pressure is applied the filter-cup is prevented from sinking too far by means of three equally spaced projections within the funnel. These projections are part of the funnel itself, being produced by indentations on its outer surface (see Fig. 1).

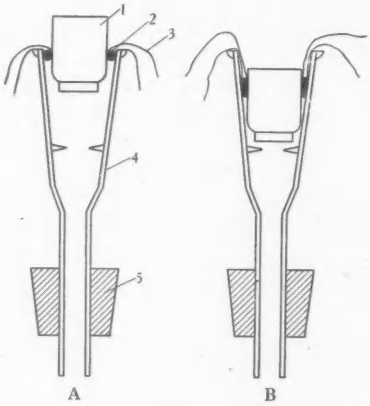

Fig. 1.

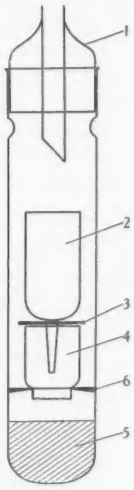

Fig. 2.

Fig. 1. Section of filter-cup and funnel. Position (A) before and (B) after application of negative pressure to pressure flask. 1, filter-cup. 2, rubber ring. 3, tape. 4, funnel. 5, rubber stopper.

Fig. 2. Apparatus for extraction. 1, reflux condenser. 2, alcohol reservoir. 3, glass rods. 4, filter-cup. 5, absolute alcohol. 6, indentations.

(6) Special extraction tube of 50 mm. diameter and approximately 270 ml. capacity. About 2 in. from its bottom it bears three indentations in its outer wall; these support the filter-cup in position. The neck of the extraction tube should be ground to fit the reflux condenser (2).

(7) 'Alcohol reservoir', represented in Fig. 2. It is about $1\frac{1}{2}$ in. in depth and only a little narrower than the outer extraction tube (6). Alcohol which collects

in it during the process of extraction can escape from its lower end through a narrow capillary tube. The alcohol reservoir is maintained in position on the top of the filter-cup by three radiating glass rods. These are fused to a point near to the origin of the capillary tube. The latter should be long enough to reach nearly to the bottom of the filter-cup when the apparatus is assembled.

Reagents

(i) For all filtrations and washings only water which has been doubly distilled in glass is used.

(ii) *The standard silver solution.* It has been found that at least 15 ml. of concentrated acid are required to complete the digestion of 10 ml. of blood to a stage when all the fatty material becomes soluble in alcohol. Therefore the *N*/20 standard solution used is prepared by dissolving the requisite amount of pure $AgNO_3$ in a minimal quantity of doubly distilled water and making up the solution to 1 l. with pure conc. HNO_3. If larger quantities of blood are used proportionately more standard solution must be taken.

(iii) *An approximately* N/*20 solution of pure NaCl* is prepared for precipitation of the excess of the standard silver solution present in the first filtrate. About 20 ml. of this are required.

Preparation of sintered glass filter cups

The required number of cups can be cleaned simultaneously in an ordinary wide-necked Erlenmeyer flask of 2 l. capacity.

The cups are gently lowered into the flask and need not be removed until all stages of preparation are completed. First a 50% solution of commercial conc. HNO_3 in tap water is added and the flask placed in a boiling water bath for 1 hr. At the conclusion of this stage the contents are removed by suction and the cups are washed several times in clean tap water.

Sufficient absolute alcohol to cover the cups is then placed in the flask, which is again set in the boiling water bath for 1 hr. The remaining alcohol is removed and the cups freely washed with doubly distilled water. Finally the flask is replaced on the water bath and the cups are heated there for 1 hr. in distilled water and washed again several times. They are then ready for drying to a constant weight.

If the cups have previously contained silver halides, it is wise as a preliminary to run a strong solution of commercial ammonia through them in the reverse direction to the usual flow. They are treated subsequently in the way already described.

DESCRIPTION OF METHOD

Stage of digestion

A measured quantity of oxalated blood (approximately 10 ml.) is carefully transferred to the special boiling tube (1). To the blood in the tube are gradually added exactly 20 ml. of the special standard solution of $AgNO_3$ in conc. HNO_3. Eisenman [1929] has found that no dissociation of halides can occur provided that the solution of strong acid and silver is added sufficiently slowly. The boiling tube is now fitted with the reflux condenser (2) and is then placed in a constant-level boiling water bath. Digestion is allowed to proceed for fully 12 hr. and after its completion the tube is found to contain a straw-coloured fluid on the surface of which are small flakes of fatty material. The white halide precipitate has sunk to the bottom.

Stage of filtration

A washed rubber ring (3) is stretched by means of three tapes passed through it. The stretched ring is lowered over a weighed filter-cup (4) which is secured by allowing the tension to relax; the tapes are left in position. The filter-cup may then be lifted by the rubber ring which grips it, and placed in the funnel (5) of a pressure flask. It is important that no contamination should occur on the sides of the filter-cups, and with this technique they are not touched at all.

The ring forms an air-tight joint between the funnel and the filter-cup. The filtration is done under a strong negative pressure. When nearly all the fluid from the boiling tube has been transferred, an assistant directs a fine jet of doubly distilled water under pressure upon the precipitate which is thus broken up into small particles. The tube is then again inclined and the contents decanted, whilst the assistant continues to wash the tube free of all particles. This is continued until no more precipitate can be seen, but as a further precaution more water is added and the tube examined against a dark background to exclude the presence of minute floating particles. With a little practice this procedure can be carried out with absolute assurance.

After emptying the tube it is further washed to remove all traces of $AgNO_3$ solution and the glass rod and cup are similarly treated.

A clean watch-glass is next placed over the top of the filter-cup; by means of gentle digital pressure on the watch-glass a sudden and violent release of the filter-cup can be prevented when the pressure-pump is disconnected. The filter-cup is now held over the funnel by the tapes of the ring to enable the assistant to wash its under surface thoroughly. The washings are carefully collected into the flask containing the filtrate. Next the filter-cup is released from the ring on to a desiccator plate, where its outer surface is further sprayed as an additional precaution. The funnel is then washed down several times to free it from all traces of $AgNO_3$. It is disconnected from the pressure-flask, which is put aside after all the $AgNO_3$ has been precipitated with excess of NaCl.

Stages of extraction

In addition to halide precipitate the cup contains flaky fatty material. This has now to be removed completely by continuous extraction with absolute alcohol in a special piece of apparatus (see Fig. 2) which consists of an extraction tube (6), an alcohol reservoir (7) and a reflux condenser (2).

The filter-cup is gently lowered by means of a long pair of forceps into the extraction tube, which contains some 30–40 ml. of absolute alcohol. The alcohol reservoir is then similarly placed in its position and the reflux condenser attached.

Extraction is allowed to proceed on a gently boiling water bath for 1–2 hr. The purpose of the alcohol reservoir is threefold. Firstly, it collects the pure alcohol as it condenses; secondly, it directs the flow of alcohol to the bottom of the cup where the fat-containing precipitate is situated; thirdly, its capillary tube exit compels a gentle flow which prevents any disturbance of the precipitate. The level of the alcohol rises in the cup and overflows, carrying the fatty material with it in solution.

The extraction is completed by allowing the water in the bath to cool, but no attempt is made to remove the filter-cup until all alcohol has left it, for fear of disturbing the precipitate. Usually 1–2 hr. are required for this to be accomplished.

The condenser is now disconnected and with the same long pair of forceps the reservoir and cup are removed. A crucible cover is then placed over the filter-cup, which is conveniently put aside on a clean desiccator plate.

Estimation of excess $AgNO_3$

The pressure-flask, containing the excess Ag which was precipitated immediately after filtration, is placed in a boiling water bath. After about an hour's heating the precipitate of AgCl will be found to have consolidated and to be in a suitable state for filtration through another specially prepared and weighed filter-cup. The procedure is exactly similar to the first filtration, except that the pressure-flask now takes the place of the digestion tube.

The two cups containing the first and second precipitates are then dried to constant weight in a hot-air oven at a temperature not exceeding 150°. On removal from the oven the cups are allowed to cool in a desiccator over phosphorus pentoxide before weighing.

Possible sources of error of the method

(*a*) *Loss of halides during the state of digestion.* Contrary to the opinion of Morris & Morris [1930], even prolonged heating of a simple halide precipitate in the presence of conc. HNO_3 does not cause any appreciable loss, as the following experiment shows.

A series of six tubes were put up, each containing 10 ml. of a standard solution of $AgNO_3$ to which had been added excess of NaCl solution and conc. HNO_3. Three of these tubes were filtered after 40 min. on a boiling water bath, but the heating in the case of the other three was prolonged for 12 hr. The weight of AgCl in the several tubes was as follows:

Duration of heating	Wt. of AgCl, g.		
40 min.	0·1416	0·1417	0·1418
12 hr.	0·1416	0·1419	0·1419

Similar conclusions were reached by Norris & Ampt [1933] who aspirated chlorine-free air through a flask containing AgCl in the presence of conc. HNO_3 at 100° and were unable to detect any trace of Cl in the fumes. Sunderman & Williams [1933], using a somewhat similar procedure, were also unable to find more than negligible amounts of halides. Further, Comey & Hahn [1921] have shown that AgBr is not decomposed by boiling with strong HNO_3.

(*b*) *Fat as an interfering constituent in halide analysis.* Sunderman & Williams [1931] have attributed a diminished recovery of chlorides to the presence of fat. These low figures were only observed when the analysis was carried out on dried, fat-containing material and with digestions by HNO_3 lasting at most 6 hr. In the present work all digestions were done wet and were allowed to proceed for at least 12 hr. In addition, it was found that the fat which resisted digestion yielded no halides on being dissolved in alcohol.

(*c*) *Technical difficulties related to the transfer of the halide precipitate to the filter-cup.* With sufficient practice this transfer can be done with great accuracy. The difficulties are therefore more apparent than real and are not a source of appreciable error. In the course of the present work many experiments have been performed to prove this, of which a small series is quoted below. The figures refer to the weight of silver halide recovered in two series of 4 and 3 parallel estimations.

Exp.	Wt., g.	Exp.	Wt., g.
1	0·0898	1	0·0936
2	0·0898	2	0·0936
3	0·0898	3	0·0936
4	0·0898		

(*d*) *Suitability of sintered glass filters used.* Personal experience has fully confirmed the claims of the makers (Schott und Gen., Jena) that the filters graded as having a porosity of 'less than 7 (No. 10 G 4)' are impermeable to silver halide precipitates, and that the less fine grades allow a proportion of the precipitate to escape through the filter.

If the cups are prepared exactly as is described above before each estimation, it appears that they retain their efficiency for a great many experiments.

(*e*) *Choice of solvent for blood-fats.* After many different solvents had been tried it was proved that with absolute alcohol it was possible to remove all fatty materials left after heating blood with HNO_3, provided that this digestion had been allowed to proceed sufficiently long. The following experiment illustrates this point. 10 ml. of blood were digested with 20 ml. of conc. HNO_3 for 12 hr., and when cool the resulting digest was filtered through a clean cup of known weight. The cup was then extracted with absolute alcohol for 1 hr. and weighed again after drying. Figures for four such experiments show that the change of weight is within the limits of experimental error.

Wt. of cup empty, g.	Wt. of cup after extraction g.
13·2053	13·2052
15·1296	15·1293
12·7384	12·7386
11·5047	11·5046

(*f*) *Solubility of silver halides in alcohol.* From the solubilities of the silver halides in alcohol at room temperature, established by Neustadt [1910], it is evident that errors from this cause are negligible.

(*g*) *Mechanical disturbance of precipitate.* During the extraction the solvent is entering the cup from above, filling it and flowing over its sides and, theoretically, it might carry with it solid particles, thus upsetting the final weight of the precipitate. In practice this criticism is not valid for several reasons. First, the capillary tube of the alcohol reservoir is made to extend nearly to the bottom of the cup and to allow a slow escape of alcohol, so that splashing is avoided. Secondly, the solvent used is of low specific gravity, whereas the silver precipitate is heavy, and even if very small particles are disturbed they immediately gravitate to the bottom. Thirdly, our own experiments, with extractions up to 12 hr. duration, prove conclusively that there is no appreciable loss. This is shown in the accompanying table.

Exp.	Nett gain or loss in wt. (g.) following extraction
1	+0·0001
2	−0·0001
3	−0·0001
4	No change
5	+0·0001
6	−0·0001
7	+0·0001

(*h*) *Solubility of silver halides in water and conc. HNO_3.* On consideration of the solubilities of silver halides in these solvents, it is evident that the amounts lost thereby must be minute.

20 ml. of conc. HNO_3 at room temperature dissolve precipitated AgCl to the extent of 0·04 mg. [Thorpe, 1872]. Similar data for AgBr are not available, but the error here is undoubtedly smaller if the relative solubilities of the two halides are taken into account.

At room temperature 100 ml. of water dissolve 0·0001462 g. of AgCl [Thorpe, 1926] and 0·0000107 g. of AgBr [Kohlrausch, 1908; Kohlrausch & Dolezalek, 1901]. Similar data for these solubilities are given by Hill [1908] and in the *International Critical Tables*. The total washings in each experiment do not exceed 200 ml.

Sample analysis

It has been found in a number of experiments, of which the following are examples, that consistent results can be obtained by this method.

Example 1. Five boiling-tubes were put up. Two of these contained 10 ml. of $AgNO_3$ solution, 10 ml. conc. HNO_3 and excess of NaCl, as controls for standardization of the $AgNO_3$ used. The three others contained 10 ml. of $AgNO_3$ solution, 10 ml. conc. HNO_3 and 10 ml. of a mixed solution of NaCl and NaBr. The halides in the mixture were then estimated by the method described. The amount of AgCl in each of the two control tubes was exactly 0·1379 g. The amounts of Cl and Br are given in the following table.

	Wt. of halide ppt., g.	
Exp.	NaCl	NaBr
1	0·0202	0·0221
2	0·0209	0·0213
3	0·0202	0·0221

Example 2. Three samples of blood were taken from a patient who had been under close observation for some weeks, and who was known not to have been taking any bromide. The total halide content of each of these was estimated with the following results:

	Wt. of halide ppt., g./100 g. blood	
Exp.	NaCl	Calculated figure for NaBr
1	0·4228	0·0006
2	0·4347	0·0002
3	0·4224	0·0008

Summary

1. A gravimetric method of estimating bromides and chlorides in organic materials has been described.

2. The possible sources of error have been considered.

3. It is claimed that the method is simple and accurate and is not open to many of the objections to which colorimetric and volumetric procedures are subject.

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109. THE DETERMINATION OF VITAMIN B_1 BY MEANS OF ITS INFLUENCE ON THE VAGINAL CONTENTS OF THE RAT

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The influence of vitamin B_1 on the vaginal contents of the mature rat has been examined with a view to using it as a criterion for the assay of that factor. Evans & Bishop [1922] showed that in the absence of vitamin 'B' from the diet, the normal cycle of changes in the contents of the vagina was upset. No keratinized cells were found and leucocytes with some nucleated epithelial cells were found day after day up to the time of death of the animal. This was confirmed by Macy *et al.* [1927]. Up to this time vitamin 'B' had not been proved to consist of several factors and our first experiment showed that this upset of the cycle of changes in the vaginal contents could be brought about by a deficiency of vitamin B_1 only. A later experiment showed that it could also be brought about by a deficiency of riboflavin only. The regular cycle could in either case be restored by giving the missing factor. The main part of the work showed that after a deficiency of vitamin B_1, the response to vitamin B_1 was graded to the dose given, i.e. the larger the dose of vitamin B_1 given the more quickly was the normal cycle of changes restored. Thus it would be possible to use this property of vitamin B_1 for its assay, but the accuracy obtainable by its use proved to be less than that obtainable by the 'rat growth' method.

Experimental

Preparation of the rats. The rats used for this work had grown to about 140 g. on the ordinary stock diet of the colony, or they had been used in vitamin A determinations (therefore never suffered from a shortage of vitamin B_1) and had then been fed on the stock diet for several weeks. Vaginal contents were examined daily by the usual 'smear method', directly, without staining, to assure ourselves that cycles were occurring normally. No rat was found that did not have cycles regularly; none had ever been given a chance of mating. As soon as a rat was found to have had three normal cycles it was given a vitamin B_1-free diet which consisted of:

Caseinogen (physiological caseinate, Glaxo) ...	15 parts
Dextrinized rice starch	79 ,,
Agar-agar	2 ,,
Salt mixture (Steenbock's 40)	4 ,,
Dried yeast, autoclaved at 120° for 6 hr. ...	25 ,,

In addition each rat was given 5 drops of a good sample of cod liver oil twice a week. Fresh tap water was supplied daily and the cages were provided with grids of $\frac{1}{2}$ in. mesh to prevent the rats from having access to their faeces.

The vaginal contents were examined daily. A rat was considered ready for experiment when no keratinized cells had been found in the vagina for 10 successive days. Most of the 107 rats used in this experiment had two or three cycles at normal intervals during the feeding of the vitamin B_1-free diet before the period of 10 days 'leucocytes only' began; only 4 rats ceased at once to have cycles. The average number of days that elapsed after the vitamin B_1-free diet was first given and the 10 days anoestrous period began was 14·25 with $\sigma = 5{\cdot}43$. 6 or 7 rats were kept together in each cage during the preparatory feeding but each rat was housed in a separate cage during the dosing period.

Dosing of the rats. As the rats became ready for the experiments, they were distributed into 6 groups of about 12 rats per group. The rats of the different groups were given daily doses of 0·01, 0·02, 0·03, 0·04, 0·05 and 0·1 g. respectively of a vitamin B_1 adsorbate that contained approximately 200 I.U. of vitamin B_1 per g. The powder was intimately mixed with finely ground dextrinized rice starch, partly to facilitate the daily measuring of doses and partly because the rats ate it more readily in this form. Each dose was given on a small dish, mixed with a little water. Vaginal smears were examined daily. When a rat had shown evidence of three cycles, it was given no further dose of vitamin B_1 until cycles had ceased and a further period of 10 days anoestrus had occurred. The whole procedure of dosing was then repeated, each rat being given the same dose as it had been given before. When its three cycles were completed for the second time it was again deprived of vitamin B_1 and the whole procedure repeated for a third time. This was done in order to determine whether the sensitivity of the rats changed with successive treatments or whether they could be used over and over again.

Results

The curve of response to graded doses of the adsorbate

It is evident (Table 1) that the responses of the different groups of rats were graded to the dose of vitamin B_1 given. Moreover, the curve of response obtained for the first series of three cycles was a steep one; but the curves obtained from the second and third series were less and less steep. Thus the rats became increasingly sensitive since they responded more readily to the dose of vitamin B_1 given. This makes it impossible to use the same rat over and over again in a determination of vitamin B_1 by this method, unless of course all the rats used in any one determination were exclusively first or second or third 'series' rats.

The curves of response were asymptotic to both axes and indicated that 0·02 g. of this adsorbate (i.e. 4 I.U.) was about the lowest dose that would bring about a series of three cycles. A dose of 0·01 g. (i.e. 2 I.U.) failed entirely to do this in any of the 7 rats to which this dose was given. In only 2 of the 7 was even one cycle produced. During an average period of 60 days of receiving this dose, the average loss of weight of the rats was 12 g.; this loss of weight was almost entirely confined to those rats which had been longest on the experiment. Thus, apparently, a minimum of 0·01 g. of this adsorbate (or 2 I.U.) is required to maintain female rats at about 140 g. weight, but it is not sufficient to maintain the normal functioning of the ovaries (as evidenced by the vaginal contents).

To test the usefulness of the curve of response, 10 rats prepared as above were given doses of 0·25 g. of a sample of dried yeast which we had previously assayed for vitamin B_1 potency by the 'rat growth' method. 9 rats were simultaneously given doses of 0·075 g. of the International Standard (adsorbate from rice polishings) for vitamin B_1. There was not enough material left to carry on the dried yeast group to the third series of cycles, but it is evident (Table 1) that

Table 1. *The length of time required to restore normal cycles in the vaginal contents of rats which had been deprived of vitamin B_1 until the anoestrous period had lasted 10 days and which had then been given graded doses of that factor, groups of about 10 rats each being used for testing each dose*

	1st series of three cycles			2nd series of three cycles			3rd series of three cycles		
Dose, g.	Aver. time days	St. dev. σ	Coeff. of var.	Aver. time days	St. dev. σ	Coeff. of var.	Aver. time days	St. dev. σ	Coeff. of var.
0·02 adsorbate product	75·6	31·1	0·412	38·6	11·7	0·304	34·0	7·1	0·208
0·03 ,,	43·7	21·0	0·480	26·8	6·0	0·223	22·8	4·7	0·205
0·04 ,,	31·4	16·2	0·516	24·2	13·6	0·560	20·9	4·7	0·223
0·05 ,,	27·0	11·1	0·410	25·1	9·4	0·373	20·6	6·4	0·312
0·1 ,,	28·3*	19·7	0·696	22·3	11·3	0·507	16·9	4·0	0·234
0·25 dried yeast	23·4	9·0	0·383	15·5	4·5	0·291	—	—	—
0·075 Int. Stand.	27·6	8·2	0·300	20·1	5·3	0·261	18·2	5·7	0·311
Average			0·457			0·360			0·249

* The time for one rat in the group was 87 days; the rest ranged from 15 to 36, average 23·0 days, $\sigma = 7{\cdot}21$, coeff. of var. $= 0{\cdot}31$. In the 2nd and 3rd series, its times were 20 and 23 days respectively.

Table 2. *Total amount of adsorbate taken per rat before three cycles were completed in each series*

	1st series		2nd series		3rd series	
Daily dose of adsorbate g.	No. of days	Total dose g.	No. of days	Total dose g.	No. of days	Total dose g.
0·02	75·6	1·512	38·6	0·772	34·0	0·680
0·03	43·7	1·311	26·6	0·798	22·8	0·684
0·04	31·4	1·256	24·2	0·968	20·9	0·836
0·05	27·0	1·350	25·1	1·255	20·6	1·030
0·1	28·3	2·830	22·3	2·230	16·9	1·690

0·25 g. dried yeast was rather more potent than 0·075 g. of the International Standard, i.e. it contained more than 30 I.U. of vitamin B_1 per g. A smooth curve was drawn through the 'first series' results, extrapolating beyond the 0·05 point, since the 0·1 point was obviously 'out'. The abscissa corresponding to the dose of 0·25 g. dried yeast was about 0·08. The abscissa corresponding to the dose of 0·075 g. International Standard was 0·045. Thus the dried yeast contained about 50 I.U. of vitamin B_1 per g. The potency obtained by the 'rat growth' method was 40 I.U. per g. In Table 1 is recorded the standard deviation (σ) for each period taken to produce three cycles. It may be seen that the values of σ decrease with decreasing times taken, so the coefficient of variation was determined for each time taken. These coefficients of variation also decrease in the successive periods, hence the responses of the rats were becoming more regular as the treatments were repeated, whether measured absolutely or relatively to the lengths of the periods (Table 1).

The time taken to produce the three cycles is inversely proportional to the dose of vitamin B_1 given, but not exactly so, which indicates that vitamin B_1 is used for other purposes than the direct restoration of normal cycles, and also, in our experiment, that 0·1 g. of the adsorbate was much in excess of the rats' needs. In Table 2 may be seen the total amounts of adsorbate needed to restore the cycles according to the different daily doses given. For the first series of cycles, apparently the most economical dose was 0·04 g. daily; for the second and third series the lowest of the doses given, 0·02 g., was the most economical

of those tried. Apparently a certain threshold value of concentration must be reached and beyond that the animal body can utilize only a proportion of the extra amount given.

The accuracy obtainable in a determination of vitamin B_1 by this method

In order to form an estimate of the accuracy obtainable by this method for determining the vitamin B_1 content of a substance, the reciprocal of the time taken by each rat to produce three cycles was used. The average for each group was calculated together with the standard deviation, σ (Table 3). The average

Table 3. *The reciprocals of times taken to restore normal cycles in the vaginal contents of rats which had been deprived of vitamin B_1 until the anoestrous period had lasted 10 days and which had then been given graded doses of that factor, groups of about 10 rats each being used for testing each dose*

	1st series of three cycles			2nd series of three cycles			3rd series of three cycles		
Dose, g.	Aver. of reciprocals of times taken	σ	Coeff. of var. %	Aver. of reciprocals of times taken	σ	Coeff. of var. %	Aver. of reciprocals of times taken	σ	Coeff. of var. %
0·02 adsorbate	0·0187	0·01424	76·2	0·0279	0·00815	29·2	0·0301	0·00629	20·9
0·03 ,,	0·0290	0·01473	50·8	0·0396	0·01209	30·5	0·0457	0·01008	22·1
0·04 ,,	0·0379	0·01406	37·1	0·0497	0·01909	38·4	0·0504	0·01320	26·1
0·05 ,,	0·0427	0·01440	33·7	0·0439	0·01265	28·8	0·0527	0·01430	27·1
0·1 ,,	0·0441	0·01636	37·1	0·0529	0·01849	34·9	0·0622	0·01462	23·5
0·25 dried yeast	0·0477	0·01476	30·9	0·0681	0·01654	24·2	—	—	—
0·075 Int. Stand.	0·0401	0·01488	37·1	0·0530	0·01431	27·0	0·0584	0·01323	22·7
Average		0·01506	43·3		0·01562	30·4		0·01311	23·7

Table 4. *Limits of error obtainable in an assay in which it is supposed that 10 rats have been used for the International Standard and 10 rats for the substance under examination*

The results from the highest dose have been omitted for it was obviously excessive.

	1st series	2nd series	3rd series
	$b=0·06142$	$b=0·04236$	$b=0·04478$
	$\sigma=0·01481$	$\sigma=0·01417$	$\sigma=0·01278$
	$\epsilon=0·006623$	$\epsilon=0·006337$	$\epsilon=0·005716$
$P=0·67$	78–128 %	71–141 %	75–134 %
$P=0·95$	61–164 %	50–199 %	56–180 %
$P=0·99$	52–192 %	41–243 %	47–213 %

values of σ for the groups producing the first, second and third series of cycles respectively were determined ($\sqrt{\{\Sigma d^2/(N-M)\}}=\sigma$ in which d = the difference from the mean of a group, N = the total number of animals used and M = the number of groups). The curves of response were approximately logarithmic and more nearly so when the result from the highest dose, which was obviously excessive, was ignored. The equations of the curves through the four points of each series were determined. From these and the average variances for the four points in each series was calculated the accuracy of a test in which 10 rats were supposed to be used for the Standard and 10 for the substance under examination, according as the first, second or third series of cycles was used (Table 4). The three series gave an average range of limits of error for $P=0·99$ of 47–216 %. This compares

unfavourably with the limits of error of the 'rat growth' method given in the British Pharmacopoeia Commission's Report of the Subcommittee on the Accuracy of Biological Assays [1936], which are 74–135% for $P=0{\cdot}99$.

As the values for σ increased almost regularly with increasing averages of times taken to produce three cycles, the coefficients of variation were determined. These also decreased in successive series for each dose of adsorbate tested.

Summary

Rats were deprived of vitamin B_1 until no keratinized cells had been found in the vagina for 10 successive days. They were then distributed in order, as they became ready, into 5 groups, and the groups were given a graded series of daily doses of 0·01, 0·02, 0·03, 0·04, 0·05 and 0·1 g. respectively of an adsorbate containing approximately 200 I.U. of vitamin B_1 per g., each rat in any group receiving the same dose.

The response was graded to the dose of vitamin B_1 given; the larger the dose the shorter was the time required to produce a series of three cycles in the vaginal contents of the rat.

When a rat had completed three cycles, vitamin B_1 was withheld until 10 successive days had passed without keratinized cells in the vagina. The rat was then given the same daily dose as before until a second series of three cycles had occurred. Then the whole process was repeated again.

The rats became increasingly sensitive to their respective doses, so that in any assay of vitamin B_1 by this method all rats used would have to be first, second or third 'series' rats, but not a mixture of the three.

One assay by this method (50 I.U./g.) of the vitamin B_1 content of a sample of dried yeast in comparison with the International Standard tested simultaneously agreed fairly well with an assay (40 I.U./g.) carried out by the 'rat growth' method.

The 'limits of error' of this method have been estimated as 47–216% for $P=0{\cdot}99$, a result which is not as good as the 74–135% 'limits of error' of the rat growth method given in the B.P.

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110. A COMPARISON BETWEEN INTERLITTER AND INTRALITTER VARIATION IN RATS WITH RESPECT TO THE HEALING OF RACHITIC BONES BY VITAMIN D

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Many workers consider that good experimentation demands the variation of only one condition at a time. This is impossible in most experiments and especially in biological assays. In these, large numbers of animals are needed and they vary greatly in many ways, even when the colony (e.g. of rats) has been fed and housed in as nearly as possible the same surroundings for many years. In order to reduce the variation to a minimum, workers have generally chosen their animals within exactly defined limits of age, weight, etc. in a vain endeavour to vary only one condition in their experiment. Fisher [1937] has forcefully pointed out the futility of this in his 'Design of Experiments' and has shown not only how information on more than one point may be obtained from a carefully planned experiment, but also how information on the interaction of influences may be obtained. We have lately applied his teaching to the arrangement of two of our experiments, one of which was primarily intended as a comparison between two preparations of irradiated ergosterol and the other as a vitamin D determination of three separate preparations. We so arranged the allocation of the doses to the rats that we were able eventually to compare interlitter with intralitter variation, a point on which most workers hold strong views without any direct evidence.

Experiment 1

Two preparations of irradiated ergosterol, A and B, thought to be of equal potency, were to be compared for vitamin D potency by means of the line test. It was decided to compare doses of 2·5, 5·0 and 10 mg. of the one with equal doses of the other, i.e. 6 doses in all. Eighteen litters (6×3) were used, each consisting of 6 rats. Sex was ignored as Coward & Key [1933] had shown that there was no correlation between sex and the healing brought about by vitamin D. The weight of the rats within each litter at the beginning of the experiment of course varied, and Coward & Key [1933] had shown that the amount of healing produced by a dose of vitamin D given after a severe rachitic condition had been developed was correlated with the initial weight. Therefore it was necessary to distribute the rats equally, not only according to the doses but also according to the initial weight. Hence in this experiment, doses in the order 10·0, 5·0, 2·5 mg. of one preparation, and 10·0, 5·0, 2·5 mg. of the other were assigned to the rats of the first litter strictly in ascending order of their initial weights. In the second litter the doses were assigned in the same order beginning with the rat of the second lowest weight, the rat of the lowest weight then receiving the

last dose of the series. In the third litter, the cycle was shifted one place again, and so on throughout the litters. In the end, the rats were distributed amongst the doses as nearly equally as possible according to their initial weights. They were equally distributed according to litters and doses. The line test was carried out in the usual way and the results are given in Table 1. All the rats in

Table 1. *Comparison of two samples of irradiated ergosterol, A and B*

2·5 mg. A		5 mg. A		10 mg. A		2·5 mg. B		5 mg. B		10 mg. B		Total healing		Aver. healing	
Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing				
52	0·75	51	1·5	50	0·375	**58**	0·375	54	1·25	53	1·5	*A*	5·750	*a*	0·96
53	0·375	51	0·75	49	1·5	49	0	**57**	0·75	55	0·25	*B*	3·625	*b*	0·60
54	0·5	54	2·0	52	2·0	51	0·75	48	2·25	**55**	1·75	*C*	9·25	*c*	1·54
59	1·5	58	1·0	56	2·25	54	0·75	53	1·25	53	2·0	*D*	8·75	*d*	1·46
52	1·0	**63**	1·5	60	3·0	59	1·0	54	1·5	52	2·25	*E*	10·25	*e*	1·71
55	2·25	49	2·75	**62**	2·5	57	1·5	56	3·5	56	3·25	*F*	15·75	*f*	2·63
55	2·5	55	3·75	52	4·25	**57**	2·0	57	2·0	56	2·5	*G*	17·00	*g*	2·83
52	1·75	52	1·75	52	1·25	51	1·5	**54**	2·25	53	2·25	*H*	10·75	*h*	1·79
59	1·0	57	2·5	57	3·25	56	1·25	56	2·75	**60**	3·25	*I*	14·00	*i*	2·33
54	2·75	51	1·0	50	2·25	49	0·75	48	1·5	48	3·75	*J*	12·00	*j*	2·00
49	0·5	**61**	2·25	58	3·5	57	1·25	55	2·0	50	1·5	*K*	11·00	*k*	1·83
48	1·0	48	1·75	**56**	3·25	54	1·0	54	2·5	53	2·5	*L*	12·00	*l*	2·00
58	3·0	57	2·5	53	2·25	**64**	3·75	64	4·5	63	3·75	*M*	19·75	*m*	3·29
57	1·0	53	2·0	52	2·75	50	1·75	**61**	2·0	61	3·0	*N*	12·50	*n*	2·08
56	1·0	56	2·5	54	1·75	49	1·5	49	1·5	**58**	2·25	*O*	10·50	*o*	1·75
59	1·0	57	1·75	56	3·5	55	2·0	54	1·75	52	2·5	*P*	12·50	*p*	2·08
48	2·0	**59**	4·5	59	2·75	57	3·0	56	3·5	52	3·75	*Q*	19·50	*q*	3·25
50	1·0	49	2·5	**56**	4·75	55	1·25	53	2·0	50	4·75	*R*	16·25	*r*	2·71
P	24·875	*Q*	38·25	*R*	47·125	*S*	25·375	*T*	38·75	*U*	46·75	*M*	221·125		
p	1·38	*q*	2·13	*r*	2·62	*s*	1·41	*t*	2·15	*u*	2·60			*m*	2·05

Pp =	34·32750
Qq =	81·4725
Rr =	123·46750
Ss =	35·77311
Tt =	83·3125
Uu =	121·55
	479·90311
Mm =	453·30625
Difference =	26·59686

Aa =	5·5200
Bb =	2·175
Cc =	14·245
Dd =	12·775
Ee =	17·5275
Ff =	41·4225
Gg =	48·11
Hh =	19·2425
Ii =	32·62
Jj =	24
Kk =	20·13
Ll =	24
Mm =	64·9775
Nn =	26·0000
Oo =	18·375
Pp =	26
Qq =	63·375
Rr =	44·0375
	504·5325
Mm =	453·30625
Difference =	51·22625

any one line or row belonged to one litter. All the rats in any one column received the same dose. Then were calculated totals and averages for each row and each column, the product of each total and average, and the sums of the products for rows and columns respectively. Then the table was rearranged, keeping all the rats of any one litter in a row but putting the healing of the heaviest rat of each litter in the first column, that of the next heaviest rat in the second column and so on (Table 2). These columns were then totalled and averaged and the products (total × average) added together. Finally, the healing obtained in each rat was squared, all the squares were added together and the

Table 2. *Healing arranged according to initial weight of rats*

	Heaviest rats ⟷ Lightest rats									
Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing
58	0·375	54	1·25	53	1·5	52	0·75	51	1·5	50	0·375
57	0·75	55	0·25	53	0·375	51	0·75	49	1·5	49	0
55	1·75	54	0·5	54	2·0	52	2·0	51	0·75	48	2·25
59	1·5	58	1·0	56	2·25	54	0·75	53	1·25	53	2·0
63	1·5	60	3·0	59	1·0	54	1·5	52	2·25	52	1·0
62	2·5	57	1·5	56	3·5	56	3·25	55	2·25	49	2·75
57	2·0	57	2·0	56	2·5	55	2·5	55	3·75	52	4·25
54	2·25	53	2·25	52	1·75	52	1·75	52	1·25	51	1·5
60	3·25	59	1·0	57	2·5	57	3·25	56	1·25	56	2·75
54	2·75	51	1·0	50	2·25	49	0·75	48	1·5	48	3·75
61	2·25	58	3·5	57	1·25	55	2·0	50	1·5	49	0·5
56	3·25	54	1·0	54	2·5	53	2·5	48	1·0	48	1·75
64	3·75	64	4·5	63	3·75	58	3·0	57	2·5	53	2·25
61	2·0	61	3·0	57	1·0	53	2·0	52	2·75	50	1·75
58	2·25	56	1·0	56	2·5	54	1·75	49	1·5	49	1·5
59	1·0	57	1·75	56	3·5	55	2·0	54	1·75	52	2·5
59	4·5	59	2·75	57	3·0	56	3·5	52	3·75	48	2·0
56	4·75	55	1·25	53	2·0	50	4·75	50	1·0	49	2·5
P	42·375	*Q*	32·50	*R*	39·125	*S*	38·75	*T*	33·00	*U*	35·375
p	2·35	*q*	1·81	*r*	2·17	*s*	2·15	*t*	1·83	*u*	1·97

$$
\begin{aligned}
Pp &= 99{\cdot}58125\\
Qq &= 58{\cdot}825\\
Rr &= 84{\cdot}90125\\
Ss &= 83{\cdot}3125\\
Tt &= 60{\cdot}39\\
Uu &= 69{\cdot}68875\\
&\overline{456{\cdot}69875}\\
Mm &= 453{\cdot}30625\\
&\overline{3{\cdot}39250}\\
\text{Difference} &= 3{\cdot}39250
\end{aligned}
$$

total healing M was multiplied by the average healing m. The difference between the sum of the squares and Mm is the total variance of the experiment. It is analysed as follows:

Sum of squares = 572·42189. Mm = 453·30625.

Difference = 119·11564.

		Degree of diff.	Mean squares	Variance ratio	Points of e^{2z}
Variance due to diff. between doses	= 26·59686	5	5·31937	11·23	0·1 % = 4·65
Variance due to diff. between litters	= 51·22625	17	3·01332	4·44	0·1 % = 2·95
Variance due to diff. between initial weights	= 3·39250	5	0·67850	1·43	5 % = 2·34
Variance due to randomization	= 37·90003	80	0·47375		
	119·11564	107	1·1039		

One would expect the variance due to differences in doses when there are two series of three each in the ratio of 1 : 2 : 4 to be significant, as indeed this calculation has shown that it is, the variance ratio 11·23 being much greater even than the 0·1 % of e^{2z} which for these numbers of animals is 4·65. The variance due to differences between litters is also highly significant, the variance ratio being 4·44, the 0·1 % point being 2·95. The variance due to initial weights is of very doubtful significance compared with the others, as its variance ratio is only 1·43, the 5 % point being 2·34. Hence there is ample evidence in this experiment that the intralitter variation in rats is much greater than the interlitter variation, when that is judged by differences in weight at the beginning of the experiment.

Experiment 2

This was a determination of the potencies of a cod liver oil and two preparations A and B made from it, each of 50 % strength. The doses tested were 40 mg. cod liver oil, 80 mg. preparation A, 80 mg. preparation B, 3 and 9 units respectively of the International Standard. They were assigned to rats in descending order of initial weight as in the first experiment. Results were tabulated in a similar way and gave the following analysis:

Sum of squares = 143·17189. Mm = 105·4878125.
Difference = 37·68408.

		Degree of diff.	Mean squares	Variance ratio	Points of e^{2z}
Variance due to diff. between doses	= 4·152	4	1·038	2·44	5 % = 2·67
Variance due to diff. between litters	= 19·4151	9	2·157	5·06	0·1 % = 4·44
Variance due to diff. between initial weights	= 0·496	4	0·124	0·29	5 % = 2·67
Variance due to randomization	= 13·621	32	0·426		
	37·684	49	0·769		

The variance due to doses was small, for four of them proved to be of nearly equal potency, only one of them, 9 units, being very different from the rest. The variance due to differences between litters was, however, highly significant, the variance ratio being 5·06, the 0·1 % point being 4·44. The variance due to differences in initial weight was again very small, the variance ratio being 0·29, the 5 % point being 2·67.

SUMMARY

It has been shown, by carefully planning an experiment designed primarily for other purposes and analysing the variance according to the method proposed by Fisher, that the variation between litters of rats in response to vitamin D is much greater than the variation between rats of the same litter as judged by their weights when they were first given the rachitogenic diet. The results emphasize very strongly the importance of using 'litter-mate' comparisons in biological assays of vitamin D.

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III. FAT-DEFICIENCY DISEASE OF RATS. THE RELATION OF THE ESSENTIAL UNSATURATED ACIDS TO TUMOUR FORMATION IN THE ALBINO RAT ON NORMAL DIET

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Rats which have been fed from the time of weaning on a diet completely free from fat gradually cease to gain in weight, and after being thus dieted for from 4 to 6 months their weight remains constant. This condition is accompanied by other symptoms, of which a dry scurfy skin and the so-called scaly tail are the most obvious [Burr & Burr, 1930]. The animals can be cured by giving them small doses of linoleic or arachidonic acids, the latter being the most potent in promoting growth. The fat-starved rat which is receiving an unlimited diet of carbohydrate, protein and the necessary accessory factors, is still able to make and store fat; indeed, the proportion of fat in rats after a very long period of fat-starvation was found to be exceptionally high [Smedley-MacLean & Nunn, 1940; Hume *et al.* 1940]. When daily doses of 7–14 mg. methyl arachidonate were given to rats which had been for many months on a fat-free diet, growth was resumed and analysis of the carcass 5 weeks later showed that the increased weight was accompanied by a diminution of the proportion of fat present when compared with animals which were still receiving only the fat-free diet. It was also observed that only a fraction of the arachidonic acid which had been administered was found in the body [Smedley-MacLean & Nunn, 1940].

The part played by the highly unsaturated acid does not appear therefore to be concerned with the formation of fat from carbohydrate or with its storage. It does, however, play some essential role in bringing about an increase in weight which is not due to the deposition of fat and which probably therefore involves the formation of new cells. The diminished store of fat found in the carcass and subcutaneous tissue when curative doses of unsaturated acids were given suggested the possibility that, in the process of growth, this diminution of fat might be directly connected with the formation of new tissue, and that the process might possibly be in some way linked up with the part played by these essential unsaturated acids.

We desired therefore to investigate the changes in the content both of fat and of highly unsaturated acid in some other condition where the formation of new cell tissue was actively proceeding. Such a condition is provided by the rapidly growing Walker tumour grafted under the skin of the rat. We therefore enlisted the co-operation of Dr Haddow to whom we are greatly indebted for providing us with the necessary material.

The rats selected were 100–150 g. in weight and were fed throughout the experiment on a diet rich in fat; it contained milk, oil cake and fish-meal so that there was a good supply of unsaturated acids. Generally speaking, in 10–14 days

after the tumour tissue had been implanted, large tumours had developed. Rats of the same strain as those into which the tumours were to be implanted and of approximately the same weight, were taken as controls and were killed at the same time that the tumours were implanted in the experimental animals. The amounts of fat and of polyunsaturated acids were estimated in the skin, liver and carcass of the controls. In some experiments a second group of controls was killed about a fortnight later at the same time as the rats bearing the tumours. The rats were not litter-mates and they varied considerably in the proportion of fat they contained.

Estimation of fat. The tumour was dissected out from its bed of fat, the liver removed, the skin with the subcutaneous tissue detached and the remainder of the carcass, exclusive of tail, head, paws and alimentary canal, minced. These fractions, tumour, liver, skin and carcass were dehydrated by immersion in acetone for at least 48 hr.; the extracts were filtered and the residues boiled with 96% alcohol for 2 hr. in an atmosphere of nitrogen. After filtering, the residual tissue was extracted with ether in a Soxhlet apparatus for 2 days; the acetone and alcoholic extracts were taken to dryness and the ether-soluble part of the dissolved matter extracted. The ethereal extracts were added together, dried with anhydrous Na_2SO_4, the ether distilled off and the total lipoid matter taken to constant weight *in vacuo*. As far as possible these operations were carried out in an atmosphere of nitrogen.

Estimation of polyunsaturated acids. The method of estimation of these acids depends on the fact that when bromine is added to their solution in ether or benzene, a mixture of isomeric bromides is formed of which only a small proportion is insoluble in ether and a still smaller proportion in benzene. These proportions however, though small, are constant.

The method of estimation adopted in our earlier experiments was as follows. A weighed amount of fat was saponified by keeping it overnight with excess of 10% alcoholic KOH; the alcohol was then distilled off, the soap dissolved in water, acidified with H_2SO_4 and the acids extracted with ether. The residue from the ethereal solution was dissolved in benzene, the solution dried with anhydrous Na_2SO_4, concentrated, ice-cooled and carefully treated with bromine until a slight excess was present. No preliminary separation into saturated and unsaturated acids was made. After 48 hr. in the cold room, the precipitate was filtered off, repeatedly washed with ether and weighed.

This method was tested by adding known quantities of arachidonic acid obtained from beef suprarenal glands to a mixture of oleic and palmitic acids in benzene solution. It gave comparative results which, though a high degree of accuracy cannot be claimed, furnish a satisfactory basis of comparison where small differences are not regarded as significant. The addition of small quantities of cholesterol did not influence the precipitation. The difficulties in the estimation of arachidonic acid have been discussed elsewhere [Dolby *et al.* 1940]. Since linoleic acid tetrabromide and linolenic acid hexabromide are soluble in benzene they are not precipitated when the precipitation is carried out in benzene solution; under this condition only the benzene-insoluble bromides of arachidonic ($C_{20}H_{32}O_2$) and clupanodonic ($C_{22}H_{34}O_2$) acids, the two acids especially characteristic of animal fat, will be precipitated. Bromine estimations made on the precipitates in these experiments gave generally between 65 and 70% Br, showing that the precipitates consisted mainly of mixtures of the octabromide of arachidonic acid and the decabromide of clupanodonic acid.

The bromide obtained from fatty acids of the livers of group IV of the tumour-bearing animals was separated by repeated extraction with hot benzene

into two fractions which were then completely analysed in Dr Weiler's laboratory. The results were as follows:

	% C	% H	% Br
Benzene-insoluble fraction	25·38	3·27	67·8
Theory for $C_{20}H_{32}O_2Br_8$	25·42	3·39	67·7
Benzene-soluble fraction	26·50	3·86	63·2
Theory for $C_{22}H_{36}O_4Br_8$	26·28	3·58	63·74

The variations in bromine estimations obtained may perhaps be partly due to the presence of polybromo derivatives of partially oxidized acids. In the experiments now described derivatives of arachidonic and clupanodonic acids were certainly present, but as the rats had been fed with a mixture containing fish-meal and oil cake, it is possible that the diet had included partially oxidized acids.

The isolation of a dihydroarachidonic acid hexabromide from the tissues of the fat-starved rat has been previously described [Smedley-MacLean & Nunn, 1940].

The method of precipitation in benzene solution has the disadvantage that the solid isomer of arachidonic bromide insoluble in benzene is only a very small proportion of the total octabromide formed and the *total* weight of bromide precipitated must be multiplied by 2·9 to arrive at the amount of arachidonic acid present. In our final experiment we precipitated the bromides in ether solution; here, the amount of ether-insoluble bromide multiplied by the factor 1·3 was taken as the weight of arachidonic present. In this case if any linolenic acid were present, which is unlikely, the bromide precipitate would also contain linolenic acid hexabromide. In any one experiment the same method of precipitation was always used for the controls and the experimental animals. The results are set forth in Table 1.

The proportion of lipoid material in the tissues of the tumour-bearing rat

The tumours lie embedded in a thick layer of subcutaneous fat. Estimations of the proportion of the total lipoid matter extracted by ether to the fat-free dry weight left after ether extraction in the various tissues, differed greatly in the control animals and even more widely in the tumour-bearing animals. Thus, in the control normal rats the percentage of lipoid to dry weight in the skin varied from 53·4 to 90·5, whilst in the tumour-bearing animals the variation was from 31 to 130. When the individual variations were so large it was obviously impossible to make any deduction unless a very large number of animals had been examined. In the carcass, the proportion of lipoid to fat-free dry weight varied from 32·6 to 41·6% in the controls and from 28·6 to 49·2% in the experimental animals. The corresponding figures for the liver were 11·5–21·1% in the controls and from 16·7 to 24·7% in the experimental animals.

The rats were fed both before and throughout the experiment on a diet rich in fat and the longest period they were allowed to survive after implantation of the tumour was 14 days. Under these conditions there was no evidence which could be regarded as significant of any change in the proportion of fat present, but as long as there was a plentiful supply both of fat and of unsaturated acid and absorption of fat from the intestine was taking place, even if increased utilization of fat occurred, it might not be apparent.

Changes in the proportion of the polyunsaturated acids (arachidonic and clupanodonic) during tumour growth

When the figures giving the proportion of highly unsaturated acid present in the skin were examined, there was a marked difference between those for the controls and those for the tumour-bearing animals.

Table 1. *Showing changes in the ratios of (1) weight of total lipoid to fat-free dry weight and (2) weight of arachidonic acid to fat-free dry weight, in the tissues of normal albino rats after the implantation of Walker tumour tissue*

	(*a*) Skin		(*b*) Carcass		(*c*) Liver	
	% lipoid dry wt.	% arachidonic acid dry wt.	% lipoid dry wt.	% arachidonic acid dry wt.	% lipoid dry wt.	% arachidonic acid dry wt.
Exp. 1:	Normal rats as controls					
4 rats killed, 9. x. 39	53·4	1·07	32·6	1·57	20·0	5·92
4 rats killed, 9. x. 39	60·6	1·19	36·2	1·95	14·7	3·86
Exp. 2:						
3 rats killed, 15. xi. 39	71·2	1·57	38·7	1·65	21·1	5·04
3 rats killed, 29. xi. 39	64·4	1·28	34·8	1·79	17·1	1·62
Exp. 4:						
3 rats killed, 29. iii. 40	79·8	1·57*	33·1	1·97*	16·9	1·83*
2 rats killed, 11. iv. 40	90·5	1·51*	41·6	1·62*	11·5	—
Mean value (calculated for 1 rat)	67·5	1·33	35·7	1·77	16·8	3·22
Exp. 1:	Rats with Walker tumours					
2 rats killed, 17. x. 39	59·7	0·93	37·2	1·42	19·6	2·53
3 rats killed, 17. x. 39 Tumours implanted, 9 x.	31·0	0·58	28·6	1·48	16·7	1·77
Exp. 2:						
5 rats killed, 29. xi. 39 Tumours implanted, 15. x. 39	44·8	0·81	31·1	1·77	24·4	5·16
Exp. 4:						
2 rats killed, 11. iv. 40	85·0	1·06*	45·0	1·62*	21·1	1·09*
2 rats killed, 11. iv. 40 Tumours implanted	130·0	0·87*	49·2	1·82*	24·7	3·41*
Mean calculated for 1 rat	61·9	0·82	36·0	1·64	21·6	3·22
Exp. 3:						
Controls for 3 rats in which tumours regressed or failed to grow	92·6	0·31	45·3	0·61	16·8	4·99

For the purpose of calculation, the unsaturated acid was regarded as arachidonic acid and its weight calculated as the total weight of bromide precipitated in benzene solution and washed with ether multiplied by 2·9 or as the total weight of insoluble bromide precipitated in ether solution (marked *), washed with ether and multiplied by 1·3.

We examined 22 control rats and 14 in which tumours were implanted. When the proportion of polyunsaturated acid to the weight of dry fat-free tissue was calculated, marked differences presented themselves (Table 1). In the skin of the tumour-bearing animals this proportion was reduced to less than two-thirds of that in the controls. In the 5 groups of controls, the values exceeded 1%, the average value being 1·33%. In 4 groups of tumour-bearing animals, the values were below 1% and in the 5th group 1·06%, the average being 0·82% and the limits of the values for the control and experimental animals did not overlap. The utilization of the essential unsaturated acid during the growth of the tumour was sufficient to reduce markedly the ratio of this acid to the dry weight of skin tissue even though the rat was supplied throughout the period of tumour growth with unlimited fat and unsaturated acids.

No such difference appeared in the carcass fat, and in the liver the results varied too widely for any conclusions to be drawn.

Table 2. *Showing weights recorded of (a) wet tissue, (b) dry tissue after ether-extraction, (c) total lipoid and (d) insoluble bromides of unsaturated acids, from normal albino rats and from those in which tumours had been implanted. The bromides were precipitated in benzene and washed with ether and their weight × 2·9 represents the weight of polyunsaturated acid calculated as arachidonic. The bromides (marked *) were precipitated in ether solution and their weight × 1·3 gives the weight of arachidonic acid. The 'dry wt.' is the weight of tissue left after extraction with alcohol and ether; the weight of ether-insoluble substance extracted by the alcohol being small. 'Lipoid' is the ether-soluble fraction consisting of fat, phospholipin and unsaponifiable matter*

Normal albino rats

			Carcass				Skin				Liver				Tumour			
Exp.	No. of rats	Wt. of rats g.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.
I	4	711·5	319·5	73·3	23·9	476	94·4	30·5	16·3	113	25·8	6·5	1·3	132	—	—	—	—
	4	502·9	261·0	59·5	21·6	431	76·3	24·7	14·9	100	24·2	5·9	0·9	79	—	—	—	—
II	3	296·9	149·6	35·8	13·8	222	51·6	15·7	11·2	85	11·6	2·8	0·6	49	—	—	—	—
	3	419·6	220·4	51·3	17·8	344	66·9	20·3	13·1	89	21·0	4·7	0·8	27	—	—	—	—
III	3	327·0	154·3	32·8	14·9	69	49·3	13·2	12·2	41	22·4	5·0	0·8	81	—	—	—	—
IV	3	326·0	155·5	33·4	11·1	508*	49·3	14·2	11·3	171*	23·1	5·7	1·0	36*	—	—	—	—
	2	280·6	131·0	27·5	11·4	342*	32·6	10·3	>9·3	119*	20·3	4·4	0·5	—*	—	—	—	—
Rats with Walker tumours																		
I	2	220	96·6	22·1	8·3	108	35·8	10·5	6·3	34	12·3	2·7	0·5	24	7·2	1·5	0·7	11
	3	350	147·2	33·1	9·5	168	44·2	15·2	5·2	30	26·6	5·9	1·0	36	33·5	3·9	0·7	19
II	5	762	326·9	73·3	22·5	449	128·6	34·6	15·5	96	45·7	9·4	2·3	168	69·6	8·1	1·3	70
III	Tumours failed to develop in experimental animals																	
IV	2	274	109·5	21·8	9·8	273*	45·6	10·0	<8·5	82*	21·1	4·6	1·0	36*	17·7	2·1	0·7	34
	2	312	125·5	26·2	12·9	360*	54·1	11·1	14·4	74*	26·8	4·8	1·2	127*	16·6	2·1	0·6	31

Estimations of Br varied from 63 to 70%.

The proportion of polyunsaturated acids present in the tumour was approximately of the same order as that present in the liver.

The results of Lavik & Baumann [1941] are interesting in this connexion. They found that the addition of fat to the basal diet of mice receiving applications of 20-methylcholanthrene to the skin increased tumour incidence from 12 to 83% and that the tumour-promoting fraction was in the fatty acid fraction. They found, however, that dietary fat was much less effective in promoting induced skin tumours in rats though the rate of tumour formation was increased by oil applied locally. Their basal diet, however, already contained a considerable proportion of fat.

Our present results confirm those which we obtained when the fat-starved rats were fed with small doses of the essential unsaturated acids and seem to establish that when new tissue is formed in the rat, arachidonic, or some similar acid, is used up during the process of growth, being drawn especially from the subcutaneous fat.

In the fat-free animals it was previously found that when increase of weight followed the administration of small doses of arachidonic acid, the proportion of fat present in the tissues fell and it was suggested that the growing cell might directly utilize stored fat in some process in which the essential unsaturated acids also played a part.

In the above-described experiments where the animals were fed throughout with a diet rich in fat, the proportion of fat in the tissues was so variable that no conclusions could be drawn.

In Exp. III (Table 1) all tumours planted in the experimental rats failed to develop or regressed. No analyses were carried out on these animals, but when the results of the analyses of the three animals which had been taken as controls were examined, they showed an exceptionally low content of arachidonic acid. The average amount of bromide precipitate from the skin and carcass fat of one rat of this group averaged 50 mg. against 100–150 mg. of the similar precipitate in controls for the animals in which tumours had grown. This result suggested the investigation of tumour-implantation in fat-starved rats where a low level of arachidonic acid exists; the particulars of this investigation are described in a subsequent communication.

Summary

Walker tumour tissue was implanted in rats of 100–150 g. weight, fed on a normal diet containing fish meal and oil cake. Large tumours developed in 10–14 days.

The proportion of lipoid substance to fat-free dry weight was determined in skin, carcass and liver and compared with the corresponding values in the controls. The figures were very variable and no significant differences were established.

The highly unsaturated acids were estimated as bromide insoluble in cold benzene and their ratio to the fat-free dry weight determined in skin, liver and carcass.

In all the tumour-bearing animals, there was a marked fall in this ratio in the subcutaneous tissue when compared with the corresponding figures for the normal control rats. No such difference was detected in the carcass and liver fat.

In the only experiment in which tumours failed to develop the controls showed abnormally low percentage ratio of highly unsaturated acid to the fat-free dry weight of the tissue.

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112. FAT-DEFICIENCY DISEASE OF RATS. THE STORAGE OF FAT IN THE FAT-STARVED RAT

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Burr & Burr [1930], to whom we owe the first description of the fat-deficiency disease of rats, originally attributed the curative effect of the essential unsaturated fatty acids to their power of promoting fat synthesis. According to these observers: 'These small emaciated animals on the fat-free diet consume as much food daily as their larger fat-containing controls yet this food is burned and none is used in growth and fat synthesis. On the addition of the unsaturated fatty acid growth is resumed and a normal amount of subcutaneous and visceral fat is found in the cured animal at autopsy. Therefore fat synthesis is dependent upon the presence in the tissues or blood stream of a certain minimum quantity of the essential fatty acids. What percentage of the growth observed is due to storage of fat and what percentage is due to increase in other compounds (protein and carbohydrate) is not yet known.'

Subsequent investigations of the respiratory quotient, at various stages of the fat-deficiency disease, carried out by Burr and his colleagues indicated that the formation of fat from carbohydrate must be taking place [Wesson & Burr, 1931; Burr & Beber, 1932]. Further confirmation of the synthesis of fatty acid from carbohydrate was supplied by an investigation of the rate of synthesis of phospholipin in the organs of the fat-starved rat. The rate of turnover of phosphorus in the liver, kidney and muscle of the fat-starved rat was as high as in those in which the fat-free diet had been supplemented with methyl linoleate; indeed in muscle tissue, the rate of phospholipin synthesis was significantly higher in those animals which had been fat-starved [Hevesy & Smedley-MacLean, 1940].

Burr & Burr recorded that the fat stores were depleted in this disease but they do not appear to have made any actual estimations of the amount of fat present. Smedley-MacLean & Nunn [1940] found an unexpectedly high percentage of fat in the skin and carcass of rats which had been fat-starved for 197 days and which had then received nine doses of methyl arachidonate; each dose was one drop of the ester weighing approximately 13 mg. given at intervals of 4 days, thus constituting an average daily dose of '$\frac{1}{4}$ drop'. The percentage of fat was actually higher in these animals than in those which had received four times as much arachidonate and in which regular growth had been resumed. At that time no estimations of the amounts of fat stored by the rat fed for a long time on the fat-free diet were available. Accepting the view that depletion of the fat stores was characteristic of the fat-starved rat, Smedley-MacLean & Nunn [1940] concluded that in their experiments the filling of the fat stores of the rats had been caused by the administration of the minute doses of methyl

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arachidonate. The diminished percentage of body fat and the increased body weight found in the rats receiving the larger doses of arachidonate were attributed to the effect of these larger doses [Hume *et al.* 1940; Smedley-MacLean & Nunn, 1940].

The present investigation was therefore undertaken to determine the condition of the fat stores in rats kept for so long on the fat-free diet that most of the highly unsaturated acids which were originally present would have disappeared.

As in previous investigations, the rats were weaned when 3 weeks old and at once placed on the fat-free diet: the technique followed was fully described in a previous communication [Hume *et al.* 1938].

Since the rats were supplied with a diet from which all ether-soluble substances had been removed the food administered could not have contained any considerable amount of choline. We did not however find that the addition of choline to the diet caused any significant improvement either in growth rate or in the general condition of the animals. The absence of choline had not therefore in these experiments acted as a limiting factor. This is in agreement with the experience of Jacobi *et al.* [1941] who found that the choline content of rats on choline-free diets increased as the body-weights of the rats increased, indicating the synthesis of choline. These authors found that in one case 76 mg. choline were synthesized by one rat in 8 weeks.

Estimation of lipoid matter. The procedure followed was that described by Smedley-MacLean & Nunn [1941] except that the head, tail and paws of the rat were minced with the rest of the carcass. The tissues examined formed approximately 85% of the total weight of the rat. When the weight of total lipoid matter (i.e. the amount of ether-soluble material extracted from the tissue by alcohol and ether) was compared with the weight of fatty acids derived from it, the fatty acid fraction comprised in the skin 95%, in the carcass about 91% of the total lipoid matter. As we were here concerned only with large differences in the amounts of fat stored, the weights of total lipoid material seemed adequate as the basis of comparison. The residual tissue after ether extraction was air-dried for 24 hr. at the laboratory temperature and its weight taken as the '*dry weight*'.

Estimation of polyunsaturated acids. The procedure described by Smedley-MacLean & Nunn [1941] was followed, except that the bromination was always

Table 1. *Showing changes in weight (g.) of rats on fat-free diet with and without supplements of linseed oil and methyl arachidonate*

Group	I	II	III	IV	V	VI	VII	VIII
No. of rats in group	1	2	2	1	3	3	3	1
No. of days on fat-free diet	172	280	280	330	197	197	197	330
Subsequent treatment	—	—	—	—	'½ drop' A for 35 days, then 37 days fat-free diet	'½ drop' A for 35 days	'½ drop' A for 68 days	Received for 240 days varying doses of arachidonate, then 0·1 g. linseed oil daily till killed
Weights of rats on 197th day	174 172	176 181	138		173 181 144	172 164 135	165 173 175	214
Change in weight from 197th day to death	−17 + 5	− 8 −16	0		+3 +7 +5	−11 − 1 + 1	−10 + 4 +11	+30

carried out in ether solution. Previous bromine estimations on similar material have shown that the insoluble bromide precipitate consisted almost entirely of the bromides of the C_{20} and C_{22} acids; the quantities of the bromides obtained were in many cases so small that estimations of bromine were not carried out on the insoluble bromide precipitates.

The proportion of lipoid material in the tissues of the fat-starved rat

The figures given in Table 2 show that the ratio of total lipoid matter to the weight of dry matter left after the ether extraction is quite as high in the carcass and skin of the fat-starved rat as in the corresponding tissue from rats fed on a

Table 2. *Showing the proportions of lipoid matter and of arachidonic acid in the tissues of fat-starved rats and in those of rats receiving supplements of esters of unsaturated acids*

Group	No. of rats	No. of days fat-starved	% wt. lipoid, dry wt.			% wt. arachidonic, dry wt.		
			Skin	Carcass	Liver	Skin	Carcass	Liver
Rats receiving only fat-free diet								
I	1	172	65·5	>32·2*	22·9	0·04	0·19	0·30
II	2	280	80·9	40·1	17·4	0·00	0·14	0·57
III	2	280	78·1	46·5	19·9	0·04	0·35	0·30
IV	1	330	93·1	30·9	20·9	0·06	0·09	0·31
Rats receiving '¼ drop' doses of methyl arachidonate after being kept for 197 days on fat-free diet only								
V	3	Dosed for 35 days, then given fat-free diet only for 37 days	86·9	29·1	16·5	0·04	0·16	0·80
VI	3	Dosed for 35 days, then killed	65·5	32·8	18·8	0·00	0·28	1·28
VII	3	Dosed for 68 days, then killed	75·3	27·2	19·4	0·23	0·31	1·28
Rat receiving, from time of weaning, with the fat-free diet a daily dose of 7–50 mg. methyl arachidonate or of linseed oil (0·1 g.) for 330 days, serving as control								
VIII	1	—	75·0	41·9	14·4	0·65	0·74	1·01

* About 1/5 of the total volume of the ethereal solution of fat was lost.

normal diet rich in fat—indeed the highest values recorded were in the fat-starved rats. The number of rats killed after a prolonged period of fat starvation without receiving curative doses was not large but it is interesting to see that in the skin of these animals, the proportion of lipoid matter to fat-free dry-weight rose as the period of fat-starvation continued.

	% lipoid to dry wt. of		
	Skin	Carcass	Liver
1 rat fat-starved 172 days	65·5	>32·2*	22·9
2 rats fat-starved 280 days	80·9	40·1	17·4
2 rats fat-starved 280 days	78·1	46·5	19·9
1 rat fat-starved 330 days	93·1	30·9	20·9

* About 20 % of the fat was lost.

It will be seen from the figures given in Table 2 that the proportion of lipoid is somewhat lower in the rats which had received the '¼ drop' doses of arachidonate up to the day on which they were killed. The one rat which had been fed with unsaturated acids in addition to the fat-free diet (group VIII) showed a high proportion of lipoid, similar to that recorded for rats on normal diet.

Table 3. *Showing actual weights recorded of (1) wet weight of tissue, (2) dry weight (fat-free), (3) total lipoid matter and (4) ether-insoluble bromide in fat-starved rats and in those receiving minimal doses of methyl arachidonate*

Group	No. of rats	No. of days on completely fat-free diet	Subsequent treatment	Skin				Carcass				Liver			
				Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.	Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.	Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.
Fat-starved rats															
I	1	172	Killed at once	27·1	8·7	5·7	2·6	104·1	27·9	>9·0	41·8	12·0	2·84	0·65	6·6
II	2	280	,,	54·5	17·2	13·9	0	215·6	58·1	23·3	63	18·0	3·84	0·67	17
III	2	280	,,	57·2	16·9	13·2	5	218·8	54·2	25·2	145?	18·4	3·67	0·73	8
IV	1	330	,,	20·3	5·8	5·4	4·9	91·5	23·6	7·3	16·5	7·3	1·53	0·32	3·7
Fat-starved rats receiving minimal doses of arachidonate															
V	3	197	35 days '$\frac{1}{4}$ drop' doses arachidonate, then 37 days on fat-free diet	81·8	24·1	20·9	8	317·4	85·8	25·0	104	27·4	6·0	0·99	37
VI	3	197	35 days '$\frac{1}{4}$ drop' doses arachidonate, then killed	75·6	23·2	15·2	0	332·8	88·6	<29·1	194	25·0	5·3	1·00	52
VII	3	197	68 days '$\frac{1}{4}$ drop' doses arachidonate, then killed	84·7	25·5	19·2	43	348·9	94·2	25·6	227	27·8	6·1	1·18	60
Fat-starved rat receiving 7–50 mg. methyl arachidonate or 0·1 g. linseed oil daily with fat-free diet, for 330 days															
VIII	1	—	—	38·7	12·0	<9·0	60	173·1	43·2	18·1	247	11·4	2·5	0·36	19

The '$\frac{1}{4}$ drop' doses of arachidonate were insufficient to produce significant increase in weight, though in the majority of the animals the loss in weight was arrested (Table 1).

The proportion of arachidonic acid in the tissues of the fat-starved rat

One of the most striking features of the estimation of the polyunsaturated acids in the fat-starved rat is that while the amount of this acid fell to a very low level during the first 6 months of fat-starvation it remained almost constant in those rats which had had varying periods of fat-starvation ranging from 172 to 330 days (Table 2, groups I–IV). This indicated that during a period of more than 6 months, the variations were almost within the limits of biological variation. Since none of this acid was being supplied, and it apparently cannot be manufactured unless some polyunsaturated acid such as linoleic is given, its rate of utilization in the second 6 months of fat-starvation must have been exceedingly small. During this period, the rat was still able to carry out the functions necessary for life but the growth of the animal ceased, the proportion of fat in the skin and carcass increased and the skin became dry and scaly, the *stratum corneum* being no longer thrown off but remaining as a hard thick layer.

Our interpretation of these results is that the small amount of arachidonic acid, so long and tightly retained in the tissues, was still sufficient to allow those functions which are essential for life to be maintained. In the process of development of new cells both to replace the worn-out cells of the body and to form the new tissues necessary for growth, the polyunsaturated acids must be utilized and some part of them is probably destroyed in the process of utilization. Smedley-MacLean & Nunn [1940] have shown that when the curative doses of the essential acids given to the fat-starved rats were sufficient to cause resumption of growth, a considerable proportion of the arachidonic acid which had been given could not be detected in the skin or carcass. Available evidence seems therefore to point to the view that a free supply of polyunsaturated acid is an essential condition for the formation of new tissue.

The rat, deprived of its essential unsaturated acid, continued to live for 6 months after growth had ceased before being killed. This behaviour is in striking contrast to that of the rat suffering from a deficiency of vitamin A or B_1 where fatal effects rapidly supervene after cessation of growth. It seems probable that a distinction must be made between those factors, a free supply of which is essential for the maintenance of the normal life of the cell and those which must be furnished when new tissue is to be formed.

It may perhaps be noted that where food for the embryo is to be provided, these acids are present in considerable quantity. It is well known that seed fats, where fat is to be utilized for the growth of the embryo, are characterized by a high proportion of linoleic acid [Hilditch, 1940]. The yolk of the egg is also rich in these acids; Nishimoto [1934], in an investigation on the phospholipins of egg-yolk, claims to have isolated a number of kephalins, rich in arachidonic acid, a di-arachidonyl kephalin being described. It is interesting that here, where material is prepared especially for the maintenance of embryo growth, it is the kephalins which are especially rich in arachidonic acid.

Feeding the fat-starved rats with very small doses of arachidonate insufficient to promote growth, improved the general condition of the rats and in the majority of cases stopped the loss of weight; under these conditions only very small changes in the proportion of this acid present in the skin and carcass were

observed; indeed any change was within the limits of variation except in the skin of the rats which had received the minimal '$\frac{1}{4}$ drop' doses for as long as 68 days where the value recorded was significantly higher. On the other hand, all rats which had received these minimal doses showed a notable increase in the proportion of polyunsaturated acid present in the liver. Whereas in the completely fat-starved rats, the proportion of arachidonic acid to the fat-free dry liver tissue lay between 0·30 and 0·57%, in those rats which had received 35 and 68 '$\frac{1}{4}$ drop' doses the value rose to 1·27%. Even in a group which, after 197 days of fat-starvation, had received 35 daily doses of '$\frac{1}{4}$ drop' arachidonate and then resumed for 5 weeks the fat-free diet, the liver value was still 0·80%. It seems from these results that the liver has some important part to play in utilizing the arachidonic acid, possibly in some process which furnishes a factor essential for the growth of new cells.

Summary

1. Rats were kept on a fat-free diet for periods varying from 172 to 330 days and the proportions of (*a*) total lipoid and (*b*) arachidonic acid to fat-free dry weight determined in skin, liver and carcass. In the subcutaneous tissue the proportion of total lipoid increased with the duration of fat-starvation.

2. The proportion of lipoid to fat-free dry weight showed no diminution when compared with the corresponding values in a rat which had received linseed oil in addition to the fat-free diet throughout the whole period. There was therefore no evidence of any diminished fat-storage in the fat-starved rat.

3. From the 6th to 11th months of fat-starvation, there was very little change in the ratio of arachidonic acid to fat-free dry weight in skin, liver and carcass.

4. The proportion of arachidonic acid to fat-free dry weight was very low in the subcutaneous tissue of the fat-starved rat. The amount of this acid in the subcutaneous tissue was not increased significantly when minimal doses of '$\frac{1}{4}$ drop' methyl arachidonate were given to rats for 5 weeks after 197 days of fat-starvation: after 10 weeks' dosing, a definite increase in the arachidonic content was detected. The change was less apparent in the carcass.

5. The level of arachidonic acid in the liver rose immediately and markedly on giving minimal doses of the curative acid even for a short period.

6. One rat received a daily dose of 7–50 mg. methyl arachidonate or of 0·1 g. linseed oil with the fat-free diet for a period of 330 days from weaning. Here the percentage of highly unsaturated acid in the skin was especially high.

7. The conclusion was drawn that a plentiful supply of highly unsaturated acid was necessary for the formation of new tissue but not for the maintenance of the normal metabolism of the cell.

8. The addition of choline to the diet produced no significant effect.

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113. FAT-DEFICIENCY DISEASE OF RATS. THE INFLUENCE OF TUMOUR GROWTH ON THE STORAGE OF FAT AND OF POLYUNSATURATED ACIDS IN THE FAT-STARVED RAT

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Since the growth of tumours in rats, fed on a normal diet rich in fat, seems to be associated with a considerable fall in the amount of highly unsaturated acids present in the subcutaneous fat [Smedley-MacLean & Nunn, 1941] there was a possibility that if the level of this acid could be sufficiently reduced, growth of the tumour might be retarded or even prevented. In previous experiments it had been found that growth of the rat on a fat-free diet ceased in 5–6 months. The level of the essential unsaturated acids would presumably be considerably reduced at an earlier date and we decided to keep rats for 14 weeks on a fat-free diet and then implant tumours. The rats chosen were 30–50 g. in weight. We again enlisted the cooperation of Dr Haddow, who implanted Walker tumour tissue both in these animals and in a set of controls which had received in addition to the fat-free diet a daily supply of linseed oil. The tumours were implanted on the 97th day of fat-starvation and the same fat-free diet was continued with and without the addition of linseed oil until the 113th day when the animals were killed and the analyses made as described in our previous communication. The results are set out in Tables 1 and 2.

Changes in the relative proportions of lipoid matter in the tissues of fat-starved rats in which tumours had been implanted

The most striking changes in the relation of lipoid matter to fat-free dry weight were those found in the skin and carcass of rats in which tumours had been implanted and had grown to large size in 14 days. These are set out in Table 1. A particularly interesting comparison was afforded by the rats in groups XI and XII which had received exactly the same diet throughout the experiment; it consisted of the basic fat-free ration supplemented by a daily dose of about 100 mg. linseed oil. After a fortnight, in 2 of the rats (group XII) the tumours had developed very slightly and seemed to be regressing. Group XI contained 5 similar rats in which large tumours had developed. The proportion of dry tumour tissue calculated on the dry weight of the total amount of tissues examined was 10 times as much per rat in the 5 rats of group XI as in the 2 rats of group XII where the tumours had failed to reach any considerable size. The fall in the proportion of lipoid to dry weight in the tumour-bearing animals was very striking (group XI, skin 44·6%, carcass 23·4%) when contrasted with the high values found in group XII (skin, 90·4%, carcass, 34·5%).

This ratio (lipoid : fat-free dry weight) was also very low in the tumour-bearing rats on fat-free diet when compared with that found in normal fat-starved

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Table 1. *Showing the percentage proportions of (1) lipoid matter and (2) highly unsaturated acid (calculated as arachidonic acid) to fat-free dry weight in the tissues of fat-starved rats and in those of rats receiving supplements of linoleic acid, after Walker tumours had been implanted*

	% Lipoid			% Arachidonic		
	Skin	Carcass	Liver	Skin	Carcass	Liver
A. Rats fed only on fat-free diet for 113 days. *Tumours* implanted on 97th day, *grew well*						
Group IX, 4 rats	42·2	26·7	16·3	0·04	0·30	0·9
Group X, 3 rats	28·6	20·3	14·4	0·05	0·33	1·3
B. Rats fed on fat-free diet with 0·1 g. linseed oil daily for 113 days. Tumours implanted on 98th day						
Group XI, 5 rats. *Tumours grew well*	44·6	23·4	17·2	0·97	1·56	4·2
Group XII, 2 rats. *Tumours remained as small nodules* and did not grow further	**90·4**	**34·5**	**24·7**	**2·33**	**1·09**	**4·1**
C. Rats fed on normal diet until tumours implanted, then transferred to fat-starved diet for 14 days						
Group XIII, 3 rats. *Tumours grew well*	37·6	19·6	22·9	0·77	2·25	5·8
Group XIV, 3 rats. Linseed oil added to fat-free diet. *Tumours grew well*	31·4	21·7	25·5	0·62	1·72	2·3
D. Normal values found in rats without tumours (for comparison)						
Rats fed on full normal diet						
Mean value	**67·5**	**35·7**	**16·8**	**1·33**	**1·77**	**3·2**
Rats fed on fat-free diet for 172–330 days						
Mean value	**82·1**	**37·1**	**18·2**	**0·03**	**0·21**	**0·4**

rats. Thus in the rats in which no tumours had been implanted the values were for the skin 65·8–98·3%, for the carcass 30·8–46·4%. When the tumours were present, the values had fallen to 28·6–42·2% in the skin and 20·3–26·7% in the carcass. The difference in these fat-starved rats is very much more striking than in previous experiments on rats which had received a normal diet, rich in both fat and unsaturated acid, during the period of tumour growth [Smedley-MacLean & Nunn, 1941].

In the liver no such sharply marked division was apparent. The figures for the tumour-bearing animals did not differ very greatly from those for the rats in which no tumours had been implanted. In the two similar groups which had received the same diet of fat-free ration with the addition of linseed oil, the proportion of liver lipoid in the resistant group was considerably higher than that of the group in which large tumours had developed, but the variations in the whole series were irregular and gave no clear indication of any special lowering of the amount of lipoid in the livers of the tumour-bearing animals.

The estimations of the total lipoid material as a basis of comparison are less satisfactory in the liver than in the skin and carcass, since the liver lipoid contains a greater proportion of ether-soluble substances other than fatty acid.

This diminution of the proportion of lipoid material (mainly fatty acid) to fat-free dry weight, so strongly marked in the skin and carcass of the rats in which actively growing tumours were present, again suggests that there is some

Table 2. *Showing actual weights recorded of (1) wet weight of tissue, (2) fat-free dry weight, (3) total lipoid matter and (4) ether-insoluble bromide in rats after implantation of Walker tumours*

Group	No. of rats			Skin				Carcass				Liver				Tumour			
				Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.	Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.	Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.	Wet wt. g.	Dry wt. g.	Wt. lipoid g.	Wt. ether-insoluble bromide mg.
IX	4	Rats received only fat-free diet; tumours implanted on 97th day and rats killed on 113th day	Tumours grew well	99·3	31·5	13·3	8·8	353·7	97·5	26·1	228	42·2	9·5	1·55	63·3	50·0	6·6	1·20	9·7
X	3			56·9	20·3	5·8	7·3	265·5	63·9	13·0	163	29·7	6·2	0·89	63·4	56·5	7·5	1·15	6·0
XI	5	Rats received in addition to fat-free diet 0·1 g. linseed oil daily throughout period of experiment; tumours implanted on 97th day. Rats killed on 113th day	Tumours grew well	111·2	33·6	15·0	252	388·7	106·9	25·0	1283	53·9	10·9	1·88	349·2	65·4	8·8	1·55	131·7
XII	2		Tumours formed only very small nodules	55·7	15·6	14·1	280	205·1	53·8	18·6	457	18·2	4·3	1·06	134·6	2·8	0·34	0·10	2·9
XIII	3	Rats of about 100–150 g. wt. which had been fed on normal diet transferred to fat-free diet for 14 days after tumours implanted	Tumours grew well	48·3	14·7	5·5	86·5	182·0	45·9	9·0	794	24·7	5·2	1·19	231·6	—	6·6	1·77	103·2
XIV	3	Rats similar to group XIII transferred to fat-free diet + daily dose linseed oil for 14 days after tumour implanted	Tumours grew well	66·2	19·7	6·2	93·5	244·8	65·9	14·3	870	29·0	6·4	1·63	111·8	—	7·0	1·72	120·2

connexion between the rapid growth of new tissue and the diminution of subcutaneous and carcass fat. A similar phenomenon had already been noted when curative doses of the essential unsaturated acids were given to fat-starved rats in which growth had previously ceased, and may possibly indicate that fatty acids have some direct part to play in the formation of new tissue, when the essential unsaturated acids are supplied. In the fat-starved rats, depletion of fat might be brought about by taking away fat from the fat stores or by inhibiting the synthesis of fat from the freely supplied carbohydrate. There is, however, no indication that there is any interference with the process of conversion of carbohydrate into fat in the fat-starved rat.

The wasting diseases in human subjects in which the fat stores become much depleted are usually associated with fever and increased metabolism or with a diminished food intake caused by starvation whether brought about by lack of food, loss of appetite or mal-absorption. It is possible, however, that utilization of fat in tumour growth might also prove a factor in bringing about the characteristic cachexia of the cancer patient. Here too depletion of the fat stores might be increased because fatty acid was being directly used to provide the growing tumour with some factor necessary for the formation of new cells.

Determinations of the respiratory quotient in slices of the liver and tumour of rats were made by Dickens & Šimer [1930] who found them to be well below unity. The mean value for liver was 0·79, for the Jensen rat sarcoma 0·81–0·84 and slices of tumours of chick, mouse, rat and man gave values from 0·81 to 0·85. The simplest explanations of the low R.Q. is that these tissues are directly burning fat and though evidence from R.Q. determinations is difficult to interpret, they at least indicate that fatty acids are directly oxidized in the tumour cell.

Changes in the proportion of highly unsaturated acid to dry weight during tumour growth

Unfortunately no control rats were available which had been starved for 113 days, the experimental period during which the rats in groups IX and X were kept on the fat-free diet. After 113 days the proportions of arachidonic acid in the skin and carcass of the tumour-bearing rats were comparable with the values found in normal rats fat-starved for the much longer period of 172–330 days. Since increase in weight continues in the fat-starved rat for 150–160 days, it is probable that there was an increased diminution in the proportion of highly unsaturated acid during the period of tumour growth, but satisfactory controls are not available to establish this. In the livers of the tumour-bearing animals the proportion of the unsaturated acid was much higher than in the animals fat-starved for the longer period.

In the two comparable groups, XI and XII, both of which comprised rats fed for 113 days on the basic fat-free diet supplemented by linseed oil, tumours grew well in the 5 animals of group XI whereas in the two animals of group XII the tumours remained as small nodules and failed to develop further. The proportions of highly unsaturated acid to dry weight in the livers of the two groups were practically identical. In the skins of the animals where the tumours had grown freely the proportion of highly unsaturated acid to dry weight of the tissue was strikingly diminished. This is in agreement with the results obtained by Smedley-MacLean & Nunn [1941] who found that when rats fed on a normal diet in which large tumours had grown were compared with normal control animals on the same diet, the proportion of highly unsaturated acid to fat-free dry weight in the skin was always diminished in the tumour-bearing animals. In the rats examined, the skin contained one-third to one-half as much fat as the

rest of the carcass (exclusive of liver and alimentary canal) so that it contained an important part of the storage fat of the whole animal; the results so far obtained suggest that when tumour growth or the restored growth of the fat-starved rat occurs it is especially in the skin that a fall in the arachidonic acid content takes place.

In the two groups XIII and XIV, tumours were implanted in rats previously fed on a normal diet rich in fat; the animals were then transferred to the fat-free diet and those in group XIII received only this until they were killed a fortnight later. The animals in group XIV received in addition a supplement of linseed oil. The values for the proportion of highly unsaturated acid to fat-free dry weight were again very low even in those which had received the linseed oil, when compared with the values found in similar rats on normal diet.

These experiments furnished no evidence therefore that a reduction of the level of the highly unsaturated acid retarded tumour growth. The tumours were implanted on the 97th day of fat starvation and the animals were killed on the 113th day. Rats, however, on this fat-free diet do not attain their maximum weight until the 150–160th day and from the 97th day to the time of maximum growth make an average increase of 26 g. per rat. Since fat-starved rats have continued to grow for 60 days and have made increases in weight up to 26 g. after the 97th day, the day on which the tumours were implanted, it is perhaps not surprising that tumour growth was not stayed. These experiments only show therefore that after 97 days of fat-starvation the diminution of the body stores of highly unsaturated acid did not prevent a further gain in weight when the rat was allowed to continue on the same diet nor did it prevent development of tumour growth after implantation of the Walker tumour. Implanted tumours grew as well in these fat-starved rats as in those which had received in addition a daily dose of 0·1 g. linseed oil. It seems possible that if the tumour were implanted after the 160th day when the level of the essential acid had fallen so low that the animal could no longer increase in weight, failure of tumour growth might then also occur; experiments are now being carried out to investigate this. So far the results suggest that as long as enough essential fatty acid is present to allow the normal fat-starved rat to increase in weight, enough is present to supply the needs of the growing tumour.

The growth of the Walker tumour in the rat seems to have some correlation with a rapid diminution of fat, especially in the subcutaneous tissue, accompanied by a marked lowering in the proportion of highly unsaturated acid to fat-free dry weight in the same tissue. The tumours in the rats which had received the essential unsaturated acids were about the same weight as those in the fat-starved rats, but they contained 10 times as much of the essential acids. Above a certain minimum therefore the presence of the acid can have no effect on the growth of the tumour.

Synthesis of clupanodonic acid from linoleic acid by the rat

Rats in the 4 groups IX, X, XI and XII received the fat-free diet during the 113 days of the experiment; those in the groups XI and XII received also 100 mg. linseed oil daily during the whole of this period and were therefore supplied with linoleic and linolenic acids. A comparison of the percentages of bromine in the ether-insoluble bromides prepared from the liver fatty acids in these four groups showed that when linoleic and linolenic acids had been supplied, the bromide stored was the decabromide of clupanodonic acid. This is shown in Table 3.

Table 3

Group	Ether-insoluble bromide from liver fatty acids mg. per rat	Br %
IX (fat-free diet)	15·8	65·1
X (fat-free diet)*	21·1	65·0
XI (fat-free diet with linseed oil)	69·8	70·5
XII (fat-free diet with linseed oil)	67·3	71·3
Theory for:		
Linoleic acid tetrabromide	—	53·3
Linolenic acid hexabromide	—	63·3
Arachidonic acid octabromide	—	67·8
Dihydroarachidonic acid hexabromide*	—	61·1
Clupanodonic acid decabromide	—	70·8

In the case of the rats fed only on the fat-free diet and which have received neither linoleic nor linolenic acids, it seems probable that the bromide stored is a mixture of arachidonic acid and its dihydro derivative, which latter was previously isolated from the liver fatty acids of the fat-starved rat as its hexabromide [Smedley-Maclean & Nunn, 1940]. In the case of the rats which had received linoleic and linolenic acids, it seems to be clupanodonic acid $C_{22}H_{34}O_2$ which has been stored and which must have been synthesized from the less highly unsaturated C_{18} acids supplied.

Summary

1. Walker tumours were implanted in (*a*) seven rats which had been maintained for 97 days on a fat-free diet, and in (*b*) seven rats which had received for the same period daily doses of linseed oil in addition to the fat-free diet. Large tumours developed in all the fat-starved rats and in five of those which had received the linseed oil. In two rats receiving linseed oil, small nodules formed but failed to develop further.

2. The five 'linseed oil rats' in which tumours had developed showed a large diminution in the proportion of total lipoid material (chiefly fatty acids) in both skin and carcass when compared with the two rats in which the tumours had remained as small nodules. The proportion of lipoid material was also much lower in the tumour-bearing fat-starved rats than in fat-starved rats in which no tumours had been implanted.

3. A marked lowering of highly unsaturated acid in the subcutaneous tissue but not in the carcass fat accompanied the development of the tumours. There was no significant change in the proportion of this acid in the liver. The fat-starved rats had not reached the stage of complete cessation of growth, so that any highly unsaturated acid available for normal growth would have been available for utilization in tumour development.

4. The ether-insoluble bromides from the fatty acids of the livers of the rats which had received linseed oil contained 70% Br, corresponding with the decabromide of clupanodonic acid $C_{22}H_{34}O_2Br$. The corresponding bromides from the livers of the fat-starved rats contained only 65% Br which would be in agreement with a mixture of arachidonic octabromide and dihydroarachidonic hexabromide. This suggests the synthesis by the rat of clupanodonic from linoleic or linolenic acid.

The microanalyses were carried out in Dr Weiler's laboratory. We desire to express our gratitude to Dr Haddow for his cooperation and to Prof. D. Keilin and Sir Charles Martin for hospitality respectively at the Molteno Institute and at Roebuck House, Cambridge.

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114. A NOTE ON THE EPIDERMIS OF THE RAT ON A FAT-FREE DIET

By RAYMOND WILLIAMSON

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THE following histological observations were made on rats described in the previous papers by Smedley-MacLean & Hume.

Sections of the skin from a rat on a full normal diet, a rat on a fat-free diet for about 11 months and a rat on a fat-free diet with a supplement of methyl arachidonate or linseed oil for the same period were examined. Two rats which had been on a fat-free diet with and without a supplement of unsaturated acid and in which tumours had been implanted and grown were also examined. There were no changes in the skins of the tumour-implanted rats, other than those produced by diet; it will therefore be convenient to describe the skin of the normal rat and those of the rats on fat-free diet and on fat-free diet with the addition of unsaturated acid respectively.

The whole animal was first placed for a short time in formaldehyde-saline and the tissues partially fixed in order to facilitate the removal of portions of tissue which could be cut at right angles to the surface so as not to produce apparent increases in the thickness of the epidermis through cutting at a slight angle from the vertical. In each case portions of skin were examined from different parts of the body, care being taken to remove them from as near the same place in each rat as possible. Every care was taken in embedding and cutting the sections to ensure that they were cut as near to a right angle from the surface of the skin as possible.

The epidermis of the rat on a normal diet was very thin, about one to two cells deep, except at the opening of hair follicles where it was slightly thicker. In the rat there is very little differentiation into layers. Next to the corium there is a layer in which one or two rows of nuclei can be seen; cell outlines are very indistinct; above this layer there is an extremely thin line of granules which represents the stratum granulosum and attached to this are a few horny flakes (Fig. 1 (*a*)).

The epidermis of the rats on fat-free diets was thicker, and showed greater differentiation than that of the normal rat. Two to three layers of cells could be distinguished with more definite cell outlines than in the normal. The nuclei of cells next to the corium stained rather more deeply than those farther away. There was a particularly well developed stratum granulosum with granules which stained intensely with haematoxylin. Above this layer there was a thick stratum corneum which was in places thicker than the rest of the epidermis. Just below the epidermis there was a slightly greater infiltration of cells than in the normal skin (Fig. 1 (*b*)).

The skins of two rats which had had fat-free diets with the essential unsaturated acids in addition were examined. In one the skin could hardly be distinguished from that of the normal rat; in the other it was intermediate in appearance between the skin of the normal rat and those on fat-free diets. In neither was the skin as thick as in rats on fat-free diets; the keratinization was less, and the stratum granulosum was also less distinct (Fig. 1 (*c*)).

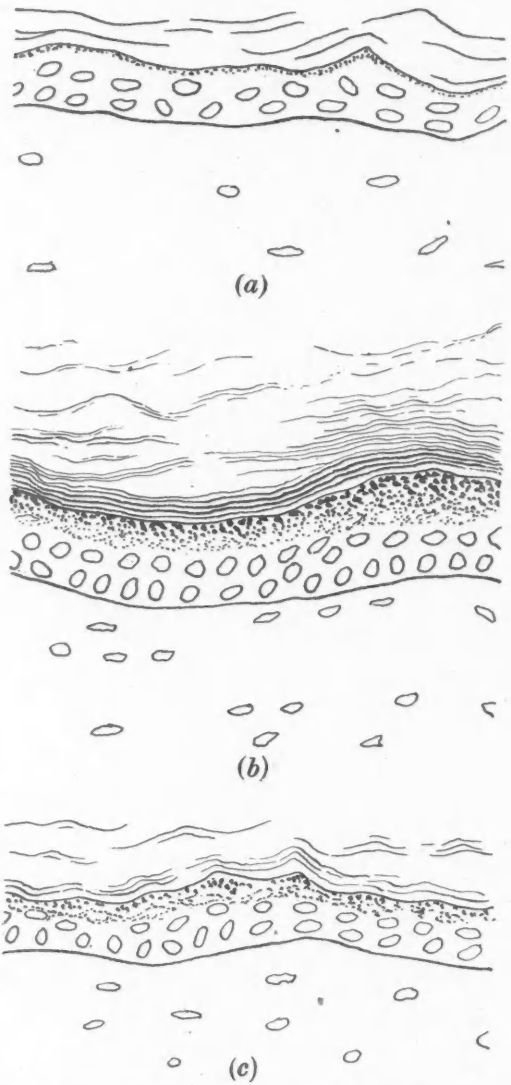

Fig. 1. Camera lucida drawings illustrating the skin of the flank of (*a*) normal rat, (*b*) rat on fat-free diet, (*c*) rat on fat-free diet plus linseed oil.

The measurements of the thickness of the epidermis excluding the horny layer were as follows:

	mm.
Normal	0·011
Rat on fat-free diet (1)	0·028
„ (2)	0·021
Rat on fat-free diet plus linseed oil (1)	0·014
„ „ (2)	0·011

Summary

The epidermis of rats on fat-free diets becomes thicker, and more differentiated than that of normal rats, the stratum granulosum becoming especially distinct and the horny layer thick. When rats were fed on fat-free diets with supplements of unsaturated acids the epidermis was found to be nearly normal.

115. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

69. SYNTHESIS OF CATENARIN (1:4:5:7-TETRAHYDROXY-2-METHYLANTHRAQUINONE), A METABOLIC PRODUCT OF SPECIES OF *HELMINTHOSPORIUM*

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(*Received 31 July 1941*)

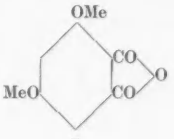
CHARLES *et al.* [1933] isolated, by chloroform extraction of the dried mycelium of laboratory cultures of the plant pathogen *Helminthosporium gramineum* Rabenhorst, two hitherto undescribed polyhydroxyanthraquinones which they named helminthosporin and hydroxy*iso*helminthosporin. Later, Raistrick *et al.* [1934] isolated hydroxy*iso*helminthosporin from *H. velutinum* Link, *H. tritici-vulgaris* Nisikado and *H. catenarium* Drechsler, and since this polyhydroxyanthraquinone occurs in only relatively small amounts in the mycelium of *H. gramineum* but is the main coloured product of *H. catenarium* it was re-named catenarin. More recently, Anslow & Raistrick [1940, 1] showed by analytical methods that catenarin is 1:4:5:7-tetrahydroxy-2-methylanthraquinone (V) and we have now confirmed this conclusion by synthesis.

Considerable difficulty was experienced before a satisfactory synthesis of catenarin was effected. Thus, although condensation of 4-methyl-3:6-dimethoxyphthalic anhydride with 1:4-dimethoxybenzene and subsequent ring closure of the resulting substituted benzoylbenzoic acid led to a straightforward synthesis of cynodontin (1:4:5:8-tetrahydroxy-2-methylanthraquinone [Anslow & Raistrick, 1940, 2], this method failed when the same anhydride was condensed with 1:3-dimethoxybenzene. A substituted benzoylbenzoic acid was readily obtained but all attempts to effect ring closure proved fruitless. This recalls a similar experience of Graves & Adams [1923] in attempting to synthesize emodin (4:5:7-trihydroxy-2-methylanthraquinone) from 3:5-dimethoxyphthalic anhydride and *m*-cresol and supports the conclusion of Jacobson & Adams [1924] that 'where, in the benzoylbenzoic acids, there is an *ortho* or *para* directing group *meta* to the position in which condensation to the anthraquinone must take place, the difficulty of forming the anthraquinone is especially pronounced'.

A successful synthesis of catenarin was finally achieved by a modification of the method used by Jacobson & Adams [1924] for the synthesis of emodin. 3:5-Dimethoxyphthalic anhydride (I) was condensed with *m*-cresol (II) and the resulting 3:5-dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl)benzoic acid (III) was brominated to give 3:5-dimethoxy-2-(2′-hydroxy-4′-methyl-5′-bromobenzoyl)-benzoic acid (IV). Ring closure of this substance was effected by heating at 100° with conc. H_2SO_4 and boric acid and the resulting condensation product, without isolation, was simultaneously demethylated and its bromine atom replaced by hydroxyl, by raising the temperature to 150–160°. Similar direct replacement of a halogen atom by hydroxyl has been previously described in the

conversion of 1-chloro-4-hydroxy-2-methylanthraquinone into 1:4-dihydroxy-2-methylanthraquinone, i.e. 2-methylquinizarin (Bayer and Co., D.R.P. 203,083; Ullmann & Schmidt, 1919].

The synthetic *1:4:5:7-tetrahydroxy-2-methylanthraquinone* agreed in all its properties with natural catenarin from *H. catenarium*.



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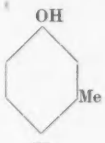

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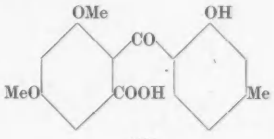

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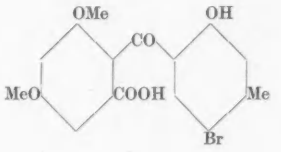

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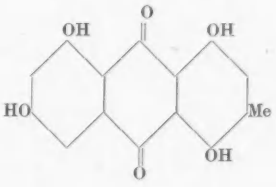

V

Experimental

The starting point of the synthesis was orcin (3:5-dihydroxytoluene). This was converted into 3:5-dimethoxyphthalic anhydride by the following stages. The yields obtained are given in brackets. (*a*) Orcin → 3:5-dimethoxytoluene (76%); (*b*) 3:5-dimethoxybenzoic acid (26%); (*c*) methyl 3:5-dimethoxybenzoate, esterification effected with diazomethane in ether (95%); (*d*) 3:5-dimethoxytrichloromethylphthalide (94%); (*e*) 3:5-dimethoxyphthalide carboxylic acid (93·5%); (*f*) 3:5-dimethoxyphthalide (93%); (*g*) 3:5-dimethoxyphthalic acid (92%); (*h*) 3:5-dimethoxyphthalic anhydride (87%). The methods used were, in general, those described by Fritsch [1897] and Graves & Adams [1923] but the following modifications were introduced with advantage.

Oxidation of 3:5-dimethoxytoluene to 3:5-dimethoxybenzoic acid. 3:5-Dimethoxytoluene (20 g.) was stirred vigorously with a hot solution of $KMnO_4$ (40 g.) in water (1 litre), and heated to boiling under reflux. After boiling for 10 min. the permanganate was completely decolorized. Unchanged dimethoxytoluene was removed by steam distillation, the hot solution was filtered and the MnO_2 was well washed with hot water. The filtrate was cooled, acidified with conc. HCl and kept at 0° overnight, when 3:5-dimethoxybenzoic acid (3·1 g., M.P. 181°) separated. Only traces of the acid remained in the filtrate. The recovered dimethoxytoluene was re-treated with $KMnO_4$. In this way 82 g. 3:5-dimethoxytoluene gave 25·9 g. 3:5-dimethoxybenzoic acid (26% of theory).

Oxidation of 3:5-dimethoxyphthalide to 3:5-dimethoxyphthalic acid. 3:5-Dimethoxyphthalide (22·45 g.) was dissolved by heating in a mixture of ethanol (250 ml.) and water (115 ml.) containing NaOH (11·5 g.). The ethanol was removed by steam distillation and to the cooled aqueous solution thus obtained there was added 3% $KMnO_4$ (850 ml.). The mixture was kept at room temperature overnight. The excess $KMnO_4$ was destroyed by adding an excess of ethanol and heating to 50°. The MnO_2 was separated by filtration, washed and

the filtrate evaporated *in vacuo* to 150 ml. After acidification with HCl the mixture was kept at 0° overnight. 3:5-Dimethoxyphthalic acid separated (24·0 g.; M.P. 155–156°; 92% of theory).

Condensation of 3:5-dimethoxyphthalic anhydride with m-*cresol. 3:5-Dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl)benzoic acid (III)*

3:5-Dimethoxyphthalic anhydride (5 g.) was dissolved in warm freshly distilled *m*-cresol (30 ml.). The solution was cooled and to it was added freshly powdered anhydrous $AlCl_3$ (10 g.) in small amounts to control frothing. The deep red mixture was kept overnight and was then heated in an oil bath at 70° for 6 hr. with mechanical stirring. The reaction mixture became darker red in colour and, after 5½ hr. heating, was very viscous and difficult to stir. After standing overnight at room temperature, ice was added together with 2*N* HCl (50 ml.) and the excess *m*-cresol was removed by steam distillation. While still hot the supernatant aqueous layer (from which 0·65 g. of 3:5-dimethoxyphthalic acid separated on cooling) was decanted. The residual oil quickly solidified and was dissolved in *N* NaOH (180 ml.) and the solution saturated with CO_2. The dull yellow phthalein by-product which separated was filtered, washed and dried. Wt. 3·1 g. Acidification of the filtrate led to the separation of the crude benzoyl acid as a brick red solid, wt. 3·3 g., M.P. 198–203°. Pure 3:5-dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl)benzoic acid was obtained from the crude product by trituration with ether (50 ml.) followed by crystallization from methanol (25 ml. + norite). The pure acid (1·45 g.; net yield 22%) was obtained as colourless rosettes of needles M.P. 233° which gave the following colour reactions: (*a*) with cold conc. H_2SO_4, orange red solution; (*b*) with $FeCl_3$ in alcohol, an intense brownish purple colour; (*c*) with *N* NaOH a bright yellow solution. [Found: C, 64·72; H, 5·12; OCH_3, 18·9%. $C_{17}H_{16}O_6$ requires C, 64·54; H, 5·10; $2CH_3O$, 19·6%.] Graves & Adams [1923] first described 3:5-dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl) benzoic acid (M.P. 233°) and claim a yield of 45% but Jacobson & Adams [1924] state 'the yields varied considerably and frequently did not exceed 20% of that calculated'.

Bromination of 3:5-dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl)benzoic acid

Pure 3:5-dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl)benzoic acid (2·43 g.) was dissolved in hot glacial acetic acid (86 ml.). The solution was immersed in a bath at 50° and to it was added, with mechanical stirring, 10% w/v solution of bromine in glacial acetic acid (13·85 ml.; calculated amount 12·28 ml.). The reaction mixture was maintained, with constant stirring, at 50° for 2 hr. The acetic acid was removed by vacuum distillation and the dry residue was refluxed with methanol (20 ml.) for 5 min. and filtered hot. Treatment of the filtrate is described below. The undissolved portion was dried, wt. 1·45 g. M.P. 256–258° and recrystallized from ethanol. 3:5-Dimethoxy-2-(2′-hydroxy-4′-methyl-5′-bromobenzoyl)benzoic acid (IV) was thus obtained as thick colourless rods; M.P. 260°, Jacobson & Adams [1924] give M.P. 258–259°. [Found: C, 51·8; H, 3·79; Br, 20·2; OCH_3, 15·6%. $C_{17}H_{15}O_6Br$ requires C, 51·64; H, 3·83; Br, 20·2; $2CH_3O$, 15·7%.] This compound gives the following reactions: (*a*) with cold conc. H_2SO_4 a dark red colour with an orange tone in thin layers; (*b*) in cold 2*N* NaOH it readily dissolved and on standing over the week-end deposited diamond shaped plates; (*c*) with $FeCl_3$ in alcohol an iodine brown colour.

Jacobson & Adams [1924] state that retreatment of the evaporated methyl alcohol filtrate, referred to above, with the calculated amount of bromine leads

to quantitative yields of 3:5-dimethoxy-2-(2′-hydroxy-4′-methyl-5′-bromobenzoyl)benzoic acid. We have been unable to confirm this statement. In our hands such treatment led to the formation of a dibromobenzoyl acid. This compound is more conveniently prepared in one stage as follows. 3:5-Dimethoxy-2-(2′-hydroxy-4′-methylbenzoyl)benzoic acid (0·5 g.) was dissolved in hot glacial acetic acid (20 ml.) and immersed in a bath at 50°. There was added, with mechanical stirring, a 10% w/v solution of bromine in glacial acetic acid (5·06 ml. i.e. twice the amount used in the preparation of the monobromo-acid). The reaction mixture was maintained with constant stirring at 50° for 2 hr., during which time pale yellow rods separated, wt. 0·64 g., M.P. 249° (efferv.). Recrystallization from ethanol gave pale yellow rectangular tablets M.P. 251°. [Found: C, 43·18; H, 3·04; OCH_3, 12·1; Br, 33·7%. Calc. for $C_{17}H_{14}O_6Br_2$; C, 43·04; H, 2·98; $2CH_3O$, 13·1; Br, 33·7%.] This compound, which is probably *3:5-dimethoxy-2-(2′-hydroxy-4′-methyl-3′:5′-dibromobenzoyl*)benzoic acid gave the following reactions: (*a*) with cold conc. H_2SO_4 a dark red colour very similar to that given by the monobromobenzoyl acid but appearing blue in thin layers; (*b*) in cold 2*N* NaOH it was almost insoluble; after standing over the week-end the liquid was full of fluffy needles; (*c*) $FeCl_3$ in alcohol, iodine-brown colour.

Preparation of 1:4:5:7-tetrahydroxy-2-methylanthraquinone (*catenarin*)

3:5-Dimethoxy-2-(2′-hydroxy-4′-methyl-5′-bromobenzoyl)benzoic acid (IV; 1 g.) was dissolved in conc. H_2SO_4 (25 ml.) containing boric acid (2·5 g.). The orange red solution was heated in an oil bath at 100° for 2¼ hr. It rapidly became iodine-brown in colour and developed a purple tint after 20 min. The temperature was raised during 30 min. to 150–160° and was maintained at this temperature for 1 hr. The solution quickly became permanganate-purple in colour, changing after 30 min. to violet with a red fluorescence.

The reaction mixture was cooled, poured on ice, diluted with water (750 ml.) and boiled for 10 min. The flocculated precipitate, after cooling, was collected by centrifuging. It was washed with water, extracted with *N*/2 Na_2CO_3 (60 ml.) and separated from a trace of insoluble matter by centrifuging. The clear violet-red solution was saturated with CO_2 and the crude 1:4:5:7-tetrahydroxy-2-methylanthraquinone which separated was collected by centrifuging. It was washed, suspended in water (50 ml.) and extracted with ether (2½ l.). Removal of the solvent from the dried ether solution yielded crude 1:4:5:7-tetrahydroxy-2-methylanthraquinone (0·19 g.; M.P. 236–242°) as a dark red solid. This was purified by sublimation in a high vacuum at 170–180°, followed by recrystallization from ethanol. *1:4:5:7-Tetrahydroxy-2-methylanthraquinone* was thus obtained as glistening, deep red, rhombic plates, M.P. 246°, alone or admixed with catenarin from *Helminthosporium catenarium* Drechsler (M.P. 246°). [Found, on material dried to constant weight in a high vacuum at 100°; C, 62·65, 62·70; H, 3·70, 3·64%. Calc. for $C_{15}H_{10}O_6$; C, 62·91; H, 3·52%.] The colour reactions of the synthetic and natural materials were identical: (*a*) with cold conc. H_2SO_4; red-violet by transmitted light and blue-violet by reflected light, becoming bluer by transmitted light on the addition of boric acid and exhibiting a slight fluorescence; (*b*) with *N* NaOH; a violet-red solution bleaching to pale yellow on standing overnight; (*c*) with *N* Na_2CO_3 and *N* NH_4OH readily soluble in the cold, violet-red solution; (*d*) $FeCl_3$ in alcohol, purple-brown colour; (*e*) glacial acetic acid, orange yellow solution with a reddish tint in thin layers and a marked fluorescence.

1:4:5:7-Tetraacetoxy-2-methylanthraquinone (*catenarin tetraacetate*). Synthetic 1:4:5:7-tetrahydroxy-2-methylanthraquinone (0·38 g.) was suspended in 4 ml. of

a mixture of acetic anhydride (50 ml.) and conc. H_2SO_4 (1 ml.) and heated at 60–70° for 30 min. On cooling, *1:4:5:7-tetraacetoxy-2-methylanthraquinone* separated. Wt. 0·35 g. M.P. 221–226°. Two crystallizations from acetic anhydride raised the M.P. to 235°, not depressed on admixture with catenarin tetraacetate from *H. catenarium* (M.P. 235°). [Found: C, 60·94, 60·88; H, 3·80, 3·90; CH_3CO, 37·8, 38·05%. Calc. for $C_{23}H_{18}O_{10}$; C, 60·77; H, 3·99; $4CH_3CO$, 37·9%.]

SUMMARY

1:4:5:7-Tetrahydroxy-2-methylanthraquinone has been synthesized. This compound was shown to be identical with natural catenarin, a metabolic product of different species of *Helminthosporium*.

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116. THE CITRIC ACID CONTENT OF ANIMAL TISSUES, WITH REFERENCE TO ITS OCCURRENCE IN BONE AND TUMOUR[1]

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THE distribution of citric acid in the animal body has not previously been systematically studied, for although Thunberg and his colleagues have made a careful examination of the citrate content of body fluids, that of the solid tissues has been largely neglected. This is all the more surprising, since Pucher *et al.* [1936] have described a sensitive colorimetric method which, unlike the enzymic one of Thunberg, is readily applicable to such material. Pucher *et al.* [1936] and Sherman *et al.* [1936] analysed only a few tissues by the colorimetric method (liver, kidney, abdominal and heart muscle of the dog), for which very low values were found (0·1–1·9 mg. citric acid/100 g. tissue). It is clear from their paper that they regarded these results as typical of the major tissues of the body, but this view is shown to be incorrect by the present work.

In contrast with these low results on solid tissues, it has long been known that several body fluids contain relatively large amounts of citrate. Thus milk contains enough for the crystallization of Ca citrate from evaporated milk, an occurrence which led to the first identification of citric acid as a normal constituent of the animal organism by Soxhlet & Henkel [1888]. Citric acid was not isolated from urine until many years later [Fasold, 1930], although it was shown to be a normal constituent by Amberg & McClure [1917], and in fact urine may also contain amounts of citric acid of similar order to that present in milk (20–120 mg./100 ml.; for literature see Östberg [1931], Scherstén [1936], Grönvall [1937], Sherman *et al.* [1936]). According to the Scandinavian workers, much less citric acid is present in cerebrospinal fluid, amniotic and follicular fluids, blood serum, aqueous humour, saliva and sweat, which contain in diminishing order about 5–0·1 mg./100 ml. Pucher *et al.* [1936] found similar low figures for blood and saliva. Finally there is the surprising observation of Scherstén [1929, 1936] that a high concentration of citric acid (up to 410 mg./100 ml.) is present in semen, and is particularly associated with the secretion of the seminal vesicles, which may contain as much as 633 mg./100 g. This brief outline of the present knowledge of citric acid distribution may be supplemented by the comprehensive reviews already quoted.

The present investigation is primarily the outcome of analyses of tumour tissue, a material of which the citric acid content has not previously been studied. The results showed that tumour frequently, though not invariably, contains a relatively high concentration of citric acid. However, certain normal organs

[1] The greater part of this work was reported to the Biochemical Society at Sheffield in Feb. 1940 [Dickens, 1940]. Its completion was delayed by difficulty in obtaining necessary replacements for the photometer used.

such as skin, hair and embryonic tissue were found to have much higher amounts than those previously reported for solid tissues. In addition, the hard substance of bone has been shown to be a hitherto unsuspected store of citric acid, which may constitute as much as 1·6% of the dry, fat-extracted bone. It follows that the skeleton is the site of by far the greatest proportion of citric acid in the vertebrate, for it occurs in high concentration in the bones of all species yet investigated.

Experimental

Methods. Freshly excised tissues were used in all experiments except those on materials such as horse hair, ox bone or dried extracted bone. The animals received their last food 18 hr. before the experiment. Immediately after dissection and weighing, the tissue was ground with sand and extracted three times with 10% trichloroacetic acid. When bone was analysed it was first chopped or pulverized, then ground with sand and digested, usually with more concentrated (40%) trichloroacetic acid at about 30°, until the hard substance had dissolved. The solid residue was then re-extracted with 10% trichloroacetic acid as above. After boiling down the combined extracts with H_2SO_4 (final conc. *ca.* *N*) and cooling, the precipitated $CaSO_4$ was removed by centrifuging.

The citric acid was estimated by the colorimetric pentabromoacetone method of Pucher *et al.* [1936], with dioxan [Johnson, 1939] instead of pyridine as the final diluent. This method of analysis is the best available, but there are some sources of error which need special attention. The fresh, filtered solution of Na_2S must be prepared immediately before use. If an aliquot part of the trichloroacetic filtrate after boiling with H_2SO_4 is taken, it is important that after dilution further H_2SO_4 is added to make about *N* concentration before the oxidation. The use of $FeSO_4$ to decolorize the $KMnO_4$ was not found consistently reliable [cf. Thunberg, 1941, 1] owing to the serious effect of even minute traces of residual Fe after washing, and instead the method of Pucher *et al.* [1934] using H_2O_2 at 6° instead of $FeSO_4$ has been found to be preferable. The addition of H_2O_2 must be carried out with all the care of a titration, a slight excess leading to rapid destruction of pentabromoacetone. Some specimens of light petroleum were unsatisfactory, as Pucher *et al.* [1936] state. Instead of using water in the blank for colorimetry, the mixture of Na_2S and dioxan was employed. By this method, accurate estimations are obtained between 0·1 and 1·0 mg. citric acid: the specificity is discussed by Pucher *et al.* [1936] and is referred to in the discussion of the present paper.

Throughout the results, citric acid is always expressed as the anhydrous substance and with the exception of dried bone, tissue weights are wet weights.

The citric acid content of tumour tissue

Table 1 shows the citric acid contents of some typical tumours. It will be seen that the animal tumours used all had large amounts, from 12 to 18 mg./100 g. These tumours, of mouse, rat and rabbit, were fairly large and varying degrees of necrosis were present. In a suitable tumour, which could be dissected into mainly necrotic and mainly non-necrotic portions, the citric acid was estimated separately in the two. The results (Table 1) show that the non-necrotic part of this Crocker mouse sarcoma contains nearly twice the concentration of the necrotic tissue. The high citric acid content is therefore a property of the tumour tissue itself, and not the result of a change, such as calcification, accompanying the necrosis. In one experiment the liver of a rat bearing a Guérin tumour was analysed simultaneously with the tumour; the liver had less than 1/5 of the

Table 1. *The citric acid content of tumour tissue*

Species	Tumour	Citric acid mg./100 g.
Rabbit	Brown-Pearce carcinoma	13·6
Rat	Walker 256 carcinoma	16·6
,,	Walker 256 carcinoma	18·4
,,	Guérin tumour	16·8
,,	Guérin (liver of same rat)	(3·0)
Mouse	Crocker sarcoma 180	14·2
,,	Crocker sarcoma 180	14·3
,,	Crocker sarcoma 180 (mainly non-necrotic)	12·3
,,	Crocker sarcoma 180 (mainly necrotic)	(7·7)
Man	Malignant dysgerminoma (seminoma) of testis	9·9
,,	Fibroadenoma of uterus	5·0
,,	Carcinoma of stomach	2·7
,,	Adjacent gastric mucosa	(2·0)
,,	Carcinoma of vulva	8·8
,,	Carcinoma of vulva	7·0
,,	Leucoplakia of vulva	8·2
,,	(Adjacent normal skin)	(5·3)

Bracketed values refer to same animal or same case.

citrate content of the tumour (Table 1). Among human tumours the results were more variable; although high values were found for the carcinoma of the vulva and seminoma, the citric acid content of the carcinoma of stomach examined was only slightly higher than that of the adjacent gastric mucosa. In one case of carcinoma of the vulva, it was possible to compare the carcinoma with the nearby leucoplakia (which may be considered as precancerous) and adjoining skin which were excised together with the carcinoma; it will be noted that the citric acid content was greater in both the precancerous and malignant tissues than in the normal skin. The fairly high value obtained for the latter may be compared with the still higher values found below for animal skin.

Comparison of the total citric acid of the tumour with that of the remainder of the animal. Since low values have hitherto been reported for the total amounts of citric acid in animal organs, it appeared that analysis might show the greater part of the citric acid of a tumour-bearing animal to be contained in the tumour. This expectation was not, however, realized. A mouse bearing a Crocker sarcoma of weight about equal to that of the animal itself was selected, and the tumour and mouse were separately analysed (Table 2). In spite of the high content of

Table 2. *Analysis of a mouse tumour and of remainder of mouse*

	Total wt. g.	Citric acid mg.	Citric content mg./100 g.
Crocker sarcoma 180	20·8	2·95	14·2
Remainder of mouse	25·2	3·62	14·4

citric acid in the tumour (14·2 mg./100 g.), this contained only 45% of the total citric acid present in mouse and tumour together. It followed that some rich source of citrate was present in the tumour-free mouse, which brought up the citrate content of the whole animal to the high level of 14·4 mg./100 g.

The possibility that the intestinal contents were responsible was excluded by analysis of another mouse receiving the same diet. This animal weighed 32·1 g. and contained a total amount of 4·17 mg. citric acid, towards which the stomach, intestines, bladder and their contents (4·1 g.) contributed only 0·253 mg.

Complete analysis of the normal animal

In an endeavour to clear up this anomaly, the analyses shown in Table 3 were made. It will be noticed that the citric acid contents reported for the rabbit, guinea-pig and mouse are on the whole somewhat higher than those of

Table 3. *Citric acid content of tissues of adult animals*

Species	Tissue	Citric acid content mg./100 g.
Rabbit	Liver	2·8
	Skeletal muscle	2·5
	Kidney	6·0
	Whole brain	4·6
Guinea-pig	Liver	1·6
	Kidney	3·9
	Whole brain	3·8
	Testis	11·5
Mouse	Skin, fur mostly removed	12·2
	Fur	133
	Seminal vesicles	128
Mouse	Fur, total citric acid	114
	Fur, hot water extract	83
Mouse	Fur	93
	Skin, fur mostly removed	9·7
Horse	Hair, unwashed	27·7*
	Hair (commercial 'washed horse hair')	21·1
Man	Sebaceous cyst from hand	11·7
	Sebaceous cyst, mainly cyst wall, with only traces of adhering contents	5·0
Mouse	Skeleton, with eyes and closely adherent connective tissue	46†
	Muscle	2·8
	Skin, fur and tail sheath	8·6
	Seminal vesicles, testes and prostate	119
	Viscera and brain	4·6

* 16·2 mg./100 g. were hot water soluble.

† As it was not possible to remove a good deal of adherent tissue, this value is too low for the skeleton alone.

dog kidney, muscle and liver (0·1–1·9 mg./100 g.) reported by Pucher *et al.* [1936]. In agreement with the results of Scherstén [1936] for other species, a high content of citric acid is present in the seminal vesicles and total male gonads of the mouse, and in the testes of the guinea-pig. Low concentrations are present in skeletal muscle of rabbit and mouse (2·5, 2·8 mg./100 g.) and in liver of rabbit and guinea-pig (2·8, 1·6 mg./100 g.), while the brains of these two species have rather larger amounts (4·6, 3·8 mg./100 g.). Kidney tissue of rabbit and guinea-pig contains considerably more (6·0, 3·9 mg./100 g.) than that of the dog (1·9, 1·2 mg./100 g. [Pucher *et al.* 1936]). The skin and hair are fairly rich sources of citric acid (cf. also human abdominal skin, Table 1). The very high content of horse hair and particularly of mouse fur was very surprising; contamination by urine or semen may be excluded as the source of this citric acid, since it occurred in both male and female mice to the same extent, and care was taken to avoid contamination. It is of interest that a large proportion of the citric acid of hair is extractable by water (200 vol., boiled with hair for 5 min., extracts 58 % of the total citrate from horse hair and 73 % from mouse fur). It seemed possible that the citric acid arose from secretion of glands in the skin, and since mice are stated not to sweat on the furry parts (in any case sweat has

only 0·1–0·2 mg. citrate/100 ml. [Scherstén, 1936]), it was thought that the sebaceous glands might be the source. This hypothesis could hardly be tested in the mouse, but analysis of a sebaceous cyst from the human hand showed the fairly high citric acid content of 11·7 mg./100 g., towards which the cyst wall contributed only a minor part (Table 3). No doubt part of the high citric acid content of the skin may be attributed to a similar origin.

In spite of its high concentration, the citric acid in the fur and skin of a mouse comprises only a small fraction (about 10%) of the total in the animal. Similar considerations apply to the seminal vesicles, which in the adult mouse might add a further 5% to the total. Even excluding these known rich sources of citric acid, there is an overall concentration in the residue of the animal of over 10 mg./100 g.

The source of the greater part of this citric acid was revealed by the complete analysis of a whole mouse after dissection (Table 4). It will be seen that about

Table 4. *Analysis of a dissected mouse*

Male adult, fed 18 hr. before, live wt. 31·2 g.

Tissues	Tissue wt. g.	Citric acid mg.	Citric acid % of total in mouse
Skeleton, with closely adherent connective tissue and eyes	8·2	3·75	69
Muscle, with dissected connective tissue and a little fat	6·8	0·19	3·5
Outer skin, including skin of tail, and fur	4·3	0·37	6·8
Seminal vesicles, testes and prostate	0·6	0·71	13·1
Liver, spleen, brain, heart, lungs, kidneys and other viscera	9·0	0·41	7·5
Total	28·9	5·43	99·9

70% of the total citric acid in the mouse is contained in the skeleton, and this provides the explanation of the remarkably high citric acid content of the whole animal.

Bone, cartilage and marrow

Bone from the foreleg of an ox was obtained from the butcher, and the marrow removed. Great difficulty was experienced in crushing the hard bone, and in consequence its solution in trichloroacetic acid was incomplete; the citric acid content found is therefore probably low. Nevertheless the result showed that while the hard substance contained at least 272 mg./100 g., the red marrow had only 41 mg./100 g. Hence the greater part of the citric acid is contained in the hard bone. A similar experiment with bone and cartilage from a kitten (Table 5) showed that the content of citric acid in the cartilage is less than 10% of that in the bony substance, including marrow. In this and in the following experiments with bone, better solution of the hard material was obtained by taking 1–2 g. quantities of fresh substance, chopped when possible, and grinding with sand, 5 g. trichloroacetic acid and 1 ml. water (or, in the case of dried, extracted bone, 0·3 g. finely powdered material with 5 g. trichloroacetic acid and 10 ml. water) and keeping overnight.

I am indebted to Mrs C. M. Burns for the specimens of bone from specially treated animals, and for kindly supplying the analyses for Ca, P and CO_2, which are included with the citric acid determinations in Table 5. For technical details of preparation and analysis reference may be made to Burns & Henderson [1935].

Table 5. *Bone analyses*

	Citric acid mg./100 g.
Fresh bone, marrow and cartilage	
Ox	
Bone of foreleg	272
Red marrow from this bone	41
Kitten (3 days old)	
Bone, including marrow, from ribs and legs	373
Cartilage, from ribs and leg joints	33
'Steamed bone meal' (ox and sheep)	1420
Dried, alcohol-extracted, powdered bone	
Pup A. Normal	1310
Pup B. After prolonged administration of parathyroid hormone	1660
Kitten C. Normal	644
Kitten D. Rachitic; low Ca diet	345

Ca and P analyses

	Blood Ca mg./100 ml.	Bone analysis (%) Ca	P	CO_2
Pup A	11·2	21·4	10·8	2·42
Pup B	13·8	20·6	10·3	2·59
Kitten C	N.D.	22·7	11·3	2·78
Kitten D	N.D.	20·0	9·59	2·43

Bone cortex was taken from the middle of the shaft, and samples are thus directly comparable in all experiments; after drying and extraction the bone was reduced to a fine powder and was dried to constant weight. The two pups, A and B (Table 5), were fed on identical diets which consisted largely of milk and porridge; B received massive and prolonged dosage with parathyroid extract. The effect of diet was studied in kittens C and D; C was fed on the usual mixed domestic diet while D received a similar diet but without milk or bones. This diet was clearly deficient in Ca, for whereas the bones of animal C although thin were not rachitic, those of D were definitely rachitic, being typically bent and having large epiphyses. The result of this experiment is discussed later in this paper, but attention may here be drawn to the increase in citrate in the bone of pup B which received parathyroid extract, and the fall in citric acid in kitten D which was on a diet deficient in Ca, and was rachitic. The great variation between the two species is also noteworthy.

The composition of steamed bone meal

The great hardness of fresh bone made it necessary, for the purpose of isolating citric acid, to find a more tractable material, which was readily available in 'steamed bone meal'. This is prepared commercially from coarsely pulverized bone, mainly of ox and sheep, which after mechanical separation from skin, hair, etc. is extracted with light petroleum and with steam, and is finally dried and reduced to a fine powder. The specimen used contained 4·20% moisture (6 hr. at 105°), 2·0% ether-extractable (mainly fat), 0·82% protein-N (*ca.* 5% protein); of the total meal 7·8% was insoluble in dilute HCl. The acid-soluble part (88·0% of the original meal, dry wt.) contained 30·3% Ca, 14·0% inorganic P and 16·05% total P, all calc. on the original wt. of meal.

The citric acid content (Table 5) was 1·42% and the CO_2 content 2·06%, both calc. on the original meal (i.e. 1·61 and 2·34% respectively, calc. on dry, fat- and protein-free, acid-soluble meal). Comparison with a typical analysis of

the 'inorganic' phase of human bone (Table 6) shows that the 'inorganic' part of the sample of bone meal used in these experiments has sustained some loss of CO_2 and Ca, but on the whole closely resembles the typical bone analysis in its mineral composition. This is regarded as evidence of the suitability of this material for comparison with natural bone.

Table 6. *Comparison of the compositions of normal bone ash and of 'steamed bone meal'*

	Bone meal, % of dry, fat- and protein-free wt.	Inorganic residue of bone (human) [Gabriel, 1894] %
CaO	48·2	51·31
P_2O_5 (inorganic)	36·5	—
(total)	41·8	36·65
CO_2	2·34	5·86
Citric acid	1·61	—
Minor constituents	—	5·92*
	93·95	99·74

* Water, 3·78; MgO, 0·77; K_2O, 0·32; Na_2O, 1·04; Cl, 0·01%.

The solubility of citric acid present in bone meal. 1 g. bone meal was shaken for 2 hr. with 100 ml. distilled water at room temperature and filtered. The clear filtrate (95 ml.) contained 0·629 mg. citric acid, or 5% of that present. Boiling with 200 ml. water extracted from the residue only a further 1·24 mg. (10% of the total). Since the solubility of $Ca_3Cit._2,4H_2O$ (Beilstein, III, 564) is 85 mg./100 ml. at 18°, equivalent to 57 mg. citric acid, it is evident that the citric acid in bone meal, if present as the Ca salt, must be in some form not readily extractable by water. The presence of appreciable amounts of free citric acid, which is in any case improbable, or of the freely soluble alkali salts seems to be excluded by this experiment.

The isolation of citric acid from bone meal

On account of the great preponderance of $Ca_3(PO_4)_2$ the isolation of a pure specimen of citric acid was accompanied by much loss; but the isolation in good yield of pentabromoacetone, as described later, appears to make further work on these extractions of citric acid hardly necessary. Two methods were used, of which the first is more laborious and more wasteful, but yields a product which is readily obtained quite pure.

Isolation by fractional precipitation. 1 kg. bone meal was extracted three times at 100° with 2·5 l. of 2*N* HCl. The filtered extract was made alkaline with NH_3 and the precipitate collected. The precipitate contained 93% and the filtrate only 5% of the citric acid initially present. The moist precipitate was thoroughly stirred with 1 l. water and 0·67 l. 18*N* H_2SO_4. After adding 1·7 l. alcohol and filtering, the filtrate was evaporated at 30° to remove alcohol; magnesium acetate (0·5 kg.) was added followed by ammonia to make alkaline. The precipitate of phosphate was removed, and to the Ca- and PO_4-free solution were added 100 g. Ba acetate and 1·9 l. alcohol. The precipitate of Ba salts was collected and washed with 70% alcohol. Three extractions of the precipitate with a mixture of 90 ml. alcohol and 10 ml. conc. HCl were combined and concentrated almost to dryness (with considerable accidental loss at this stage). The residual syrup was treated with 50 ml. 10% $CaCl_2$ and the boiling solution made alkaline with NH_3. The Ca salt, after washing and drying, weighed 2·15 g.; it

was freed from Ca by the calculated amount of N H_2SO_4 in 50% alcohol. The syrup remaining after evaporation on the water bath was dried; yield 0·55 g. It was recrystallized, with a trace of animal charcoal, from ether by refluxing with 60 ml. for 6 hr., evaporating the greater part of the ether, and keeping in a stoppered flask. The sparkling white crystals were washed twice with ether. Yield 0·325 g., M.P. (slow heating) 153·0° corr., mixed M.P. 153·5° corr., M.P. of authentic anhydrous citric acid 153·5° corr.

Direct extraction after acidification. 95 g. bone meal were extracted with ether for 3 hr. in a Soxhlet apparatus to remove fat. The dried residue was cooled in ice and well stirred with 95 ml. 7N H_2SO_4. Three ether extractions of this paste, each of 24 hr., were combined. The yields of citric acid in each, by analysis, were 31, 26 and 3·0% of the total present. The extract was shaken with $NaHCO_3$ and the alkaline extract acidified with HCl; CO_2 and dissolved ether were removed on the water bath, and after boiling with animal charcoal the citrate was precipitated by addition to the boiling solution of 35 ml. 25% $CaCl_2$ and NH_3 to make alkaline. The precipitate of Ca salt was collected, washed twice with boiling water and dried. Yield 1·778 g. Ca salt containing by analysis 0·749 g. citric acid, or 60·2% of that present in the original meal. The Ca salt was ground with H_2SO_4 (16·6 ml. N) in 50% alcohol, filtered, and the precipitate re-extracted with 20 ml. 50% alcohol. The combined extracts were evaporated; the residue was dried in the oven, dissolved in a little alcohol (later experience showed this to be undesirable) and refluxed with ether. After removal of some insoluble material, the evaporated ether solution largely solidified on long keeping in a vacuum desiccator to a yellow crystalline residue (0·743 g.) which, however, still contained some alcohol. It was redissolved, with a trace of animal charcoal, in 20 ml. ether, filtered, evaporated and dried. Yield of nearly pure citric acid 0·502 g. = 40% of that calculated from analysis of original bone meal. The pale yellow crystals after one recrystallization from anhydrous ether yielded pure white citric acid, M.P. 152–153° corr.

Preparation of pentabromoacetone from bone meal

The following is based upon the preparative methods described by Wöhlk [1902] and Grönvall [1937]. 50 g. bone meal, containing by analysis 0·620 g. citric acid, were ground with a solution of 170 g. trichloroacetic acid in 100 ml. warm water. Rapid solution of all but a few coarse bony particles occurred, and after 30 min. the liquid was separated by filtration and centrifuging. To the filtrate cooled in ice 110 ml. 18N H_2SO_4 were added (temp. $<15°$). The precipitate of $CaSO_4$ was well washed with water and the combined filtrate and washings (700 ml.) were evaporated over a flame to 500 ml.; two lots of 750 ml. water were added, boiling down to 500 ml. again after each addition, to destroy trichloroacetic acid. After keeping overnight in the ice-box, the clear solution was filtered from $CaSO_4$ and some fatty material.

5 g. KBr were added, and the solution warmed to 30° while 50 ml. 5% $KMnO_4$ were added. During 30 min. at 30° three lots of 10 ml. 5% $KMnO_4$ were added at intervals; the mixture was then decolorized with 30% $FeSO_4$, when a white crystalline precipitate appeared immediately, and more separated overnight at 0°. Yield 1·165 g. of nearly pure pentabromoacetone, M.P. 68–70° (equivalent to 0·495 g. citric acid or 80% of that found by analysis of the original bone meal). After one recrystallization from a little 50% alcohol, brilliant white crystals, M.P. 73°, mixed M.P. 73°, M.P. of authentic pentabromoacetone 73°, were obtained.

Citric acid in the embryo

The embryo is of particular interest in connexion with citric acid, because the mainly carbohydrate metabolism of the young embryo [Dickens & Šimer, 1930] and the later extensive calcification are both processes in which citric acid may be expected to play a part. Table 7 gives some preliminary results on this

Table 7. *Citric acid content of the embryo*

Species	Age	Average wt. of one embryo g.	No. of embryos for analysis	Citric acid	
				mg. per embryo	mg./100 g. embryo
Mouse	—	0·9	9	0·112	12·4
Mouse	New-born—a few hr. old	1·2	7	0·194	16·2
Chick	3½ days	0·037	6	(0·012)*	(31·0)*
	5 ,,	0·217	6	0·059	27·4
	8 ,,	1·263	2	0·168	13·3
	12 ,,	5·60	1	0·740	13·2
	21 ,,	48·60	1	4·56	9·4

* At lower limit of accuracy of the method.

material, from which it appears that an unusually high content of citric acid is indeed a feature of embryonic tissue. This is particularly marked in the earliest stage which can be readily investigated with a method of the sensitivity of the present one (3½- to 5-day chick); thereafter up to the 12th day the citric acid falls to a level comparable with that of the more advanced mouse embryo, the new-born mouse, and indeed the adult mouse (cf. Table 4). In comparing these results with the adult values, however, it must be remembered that 70% of the citric acid of the adult mouse is contained in the skeleton. The determination of the distribution of citric acid in the organs of the embryo should therefore be particularly informative. This is also a necessary step before any useful comparison with the citric acid content of tumours, that is of normal and pathological growth, can be made.

Discussion

The main result of this investigation is the discovery of citric acid as a major constituent of normal bone. The preliminary estimations [Dickens, 1940] have now been confirmed for the bones of several animals by Thunberg [1941, 1], who found by the colorimetric method that the bone substance of all species studied (horse, ox, sheep, pig and also the hen and 5 species of fishes) contained citric acid amounting to '1 bis einige Prozent der Trockensubstanz'. He also found about 0·5% of citric acid in the teeth of the cat, ox and pig. These results, together with those on the bones of the mouse, ox, kitten and puppy and on mixed bone meal which are contained in the present paper, show that the presence of a high concentration of citric acid is a very widespread, probably general, property of bone.

That the substance estimated is really citric acid, and not some unknown constituent giving a similar colorimetric reaction, is shown first by the isolation of pure citric acid from bone meal and secondly by the isolation of pure pentabromoacetone in 80% of the yield calculated from colorimetric analysis. This latter proof may reasonably be taken to mean that practically the whole of the substance is citric acid, for the isolation would not be expected to be entirely quantitative, and the formation of pentabromoacetone is such an unusual reaction, depending as it does on the intermediate formation of the enolic form of $\alpha\alpha'$-acetonedicarboxylic acid, that it is very highly specific for citric acid [cp.

Grönvall, 1937, pp. 62 *seq.* for literature]. Tartaric, succinic, malic, lactic, oxalic, pyruvic, camphoglycuronic, uric, tricarballylic, *trans*aconitic, itaconic, citraconic, and mesaconic acids are stated not to interfere, nor do glycerol, sucrose, glucose, $CaSO_4$ or $Ca_3(PO_4)_2$. Pucher *et al.* [1936] add to this list acetone, glycogen, acid haematin, creatine, creatinine, cholesterol, amytal, urea, taurine, acid digest of casein, allantoin; hippuric, maleic and fumaric acids: they found, however, that β-hydroxybutyric gave about 1/1000–1/200 and the ethyl ester of acetone-dicarboxylic acid about 1/110 of the colour given by citric acid. *cis*Aconitic acid gives no colour [Johnson, 1939]. From these data it must be concluded that there is no known physiologically occurring substance other than citric acid which can be held responsible for the pentabromoacetone prepared from bone.

The fact that so large a quantity of a familiar substance has hitherto escaped detection by bone analysis is surprising, but it is upon bone ash that the most accurate and complete analyses have been made, and in these the citrate would presumably appear as an addition to the carbonate fraction. It has long been recognized that the nature of the organic combination of bone cations is an almost unknown field, and a systematic study of bone for the presence of other physiologically important organic acids would now appear profitable.

Since the citric acid of bone meal is insoluble in alcohol and ether and is only slightly extracted by water, it is assumed that it may be present in combination with calcium, though this combination may be in the form of a complex ion, such as citrates are known to form with Ca [cf. Hastings *et al.* 1934]. The nature of its distribution throughout the bone, and its possible entry into the mixed crystal structure of bone, are questions for future investigations.

The mere presence of the highest concentration of citrate in that tissue which is richest in calcium, however, seems to provide by far the most striking evidence for a participation of citric acid in calcium metabolism. The idea of a citrate-like substance acting as a solvent for bone salts is a very old one [cf. Klinke, 1928], and Greenwald [1926] in his studies of parathyroid activity went so far as to postulate the formation of such a substance directly or indirectly by the parathyroid gland. He actually tested for citrate, presumably in the blood, but with negative results [Greenwald, 1926]. Thunberg and his associates have continually referred to the association of citric acid with calcium in the body, a striking example being the high content of citrate in semen, which has some 50 mg./100 ml. of Ca, about 5 times the concentration in plasma, and also has a high concentration of inorganic phosphate, without the precipitation of Ca occurring [Scherstén, 1936]. The high citric acid contents of skin and embryo, reported in the present paper, may be considered as strengthening this connexion, for skin has on a dry weight basis 'considerably more Ca and less Mg and P than in most other soft tissues' [Schmidt & Greenberg, 1935]; the case of embryo is discussed below. Recently, Thunberg [1941, 1, 2] has demonstrated the presence of 0·08–0·35 % of citric acid in the shell of eggs from many species of birds.

Since it is now well recognized that bone substance is continually in a state of vigorous metabolic exchange with body fluids [cp. Hevesy *et al.* 1940] it may be expected that the citrate of bone will accompany the other bone salts during their mobilization and deposition. Thus the stores of citrate in the bone will probably be available for the metabolic requirements of body tissues in general, whether catalytically in carbohydrate oxidation [Krebs & Johnson, 1937] or as solvent for Ca salts in Ca metabolism. In particular, it seems probable that the presence of citrate in the immediate vicinity of calcifying or decalcifying bone might, by its well-known solvent action, play an important part in preventing premature precipitation of bone salts in this region.

The present evidence is certainly far too meagre to decide if this mechanism is really in operation or not. Some tentative support for a participation of bone citrate in calcification may, however, be claimed from the analyses of Table 5. On the basis of these individual results, it appears that the bone citrate is considerably affected by both hormonal and dietary conditions. In fact, it is much more affected than the other salts of bone, as is clearly shown by the recalculation of the data of Table 5 in terms of the molecular proportions of the three main constituents (Table 8). On the basis of these figures, parathyroid administration,

Table 8. *Molar ratios in dried extracted bone*

	Ratio of atoms (or mol.) of				Estimated molecular proportions* of		
	Ca	P	CO_2	Citric acid	$Ca_3(PO_4)_2$	$CaCO_3$	$Ca_3Cit._2$
Pup A, normal	78·3	50·8	8·04	1	100	33	2·1
Pup B, parathyroid	59·5	38·4	6·80	1	100	38	2·7
Kitten C, normal	198·5	108·2	18·8	1	100	32	0·9
Kitten D, rickets	278·2	172	30·8	1	100	37	0·6
(Dried bone meal	116	69·4	7·06	1	100	20	1·4)

* Approximate only, based on the assumption that all the P is present as $Ca_3(PO_4)_2$, all CO_2 as $CaCO_3$ and all citric acid as $Ca_3Cit._2$.

tending to hypercalcaemia, increases the relative molar proportions of citrate to $Ca_3(PO_4)_2$ and, to a less extent, to $CaCO_3$. On the other hand, a diet deficient in calcium has the opposite effect: the citrate is diminished by some 30%. The weakness of this evidence needs no emphasis; the normal variation has not been taken into account, and in the second experiment the diet deficient in Ca, from which milk and bones were omitted, was almost certainly also low in citric acid content. It shows, however, that the variations in citrate do greatly exceed those of the other salts; further experiments are being undertaken by Mrs Burns and it is hoped that these may provide more definite evidence. In this connexion, attention may be drawn to the calcifying action of citric acid and of citrates when added to a rachitic diet [cf. Hathaway & Meyer, 1939; Day, 1940].

The embryo. It is instructive to compare the data of Table 7 for the citric acid content of the chick embryo with those of Mankin [1929] on the Ca content (Table 9). Calculation of the ratio of equivalents of Ca to those of citric acid

Table 9. *Relationship of Ca to citric acid in the embryo chick*

Age in days	Average wt. g.	Ca, mg./100 g. embryo wet wt. [Mankin, 1929]	Citric acid mg./100 g.	Equiv. Ca / Equiv. Cit.
$3\frac{1}{2}$	0·037	—	31·0	3·3 : 1
4	0·144	32	—	
5	0·217	—	27·4	3·9 : 1
5	0·183	33	—	
8	1·263	—	13·3	5·8 : 1
8	1·010	24	—	
12	5·6	—	13·2	18·8 : 1
12	5·82	77·5	—	
21	—	381	9·4	130 : 1

(column 5) reveals the remarkable fact that in the earliest stages capable of investigation by the present method (4–5 days), the citrate present in the whole embryo is sufficient to combine with almost 1/3 of the total Ca. Thereafter, the total Ca increases much more rapidly than the citric acid.

The presence of so high a concentration of citrate relative to Ca, may, if it proves to be a general feature of embryonic metabolism, be of great importance in securing an adequate supply of readily mobilizable Ca for the use of the very young embryo.

It seemed probable that the shell and contents of the hen's egg would contain enough citric acid to provide all that contained in the embryo, for originally the shell of a 50 g. egg has 7·5 mg., the yolk 2·3 mg. and the white 0·3 mg. [Thunberg, 1941, 2]. In fact a newly hatched chick (Table 7) from an egg, which before incubation weighed 64·2 g., contained 4·56 mg. citric acid, or 3·55 mg. calculated for a 50 g. egg. Unincubated eggs of the same strain (R. I. R) contained, per 50 g. egg, 3·45 mg., and 21-day incubated, non-fertile, eggs 3·87 mg. citric acid (means of 3 each); while the shells of these eggs had only 2·18 and 1·95 mg., a much lower proportion than in Thunberg's experiments. The shell at hatching had 1·55 mg. in the example quoted in Table 7.

Equally important for the question of the origin of citric acid in the body, is a reconsideration of the work of Sherman *et al.* [1936] in the light of the present results. These authors conclude that 'consideration of the large amounts of citrate which are excreted by dogs on low citrate diet during prolonged alkalosis, and the absence of stores of preformed citrate in blood, liver, muscle and kidney, lead to the conclusion that the dog can synthesize citric acid'. Examination of the results of Sherman *et al.* [1936] shows that the three dogs studied by them excreted, over periods of from 18 to 45 days, 392, 335 and 608 mg. citric acid per kg. of dog. Such large amounts could not arise from preformed stores in tissues other than bone, but it seems possible that they might originate in the skeleton. Data for the dry weight of the total bones of the dog have not been found, but in a well fed rabbit the bones constitute 8·7% of the body wt. [Aron, 1909, p. 196]. Assuming a similar value for the dog, this animal would have per kg. some 80–90 g. dry bone. If we take the value from Table 5 for the normal pup, as applying to the whole bone of the dog, and make an allowance for the fat content, these bones might have about 1% citric acid. If these figures prove to be correct, the skeleton alone of dogs would contain about 800–900 mg. citric acid per kg. of dog, more than enough to cover the whole excretion of citrate observed by Sherman *et al.* [1936], provided that the loss of citric acid from the bones could reach 60% of the total amount originally present. From this admittedly very rough calculation it appears doubtful if the evidence of these authors, unsupported by bone analyses, may be interpreted to prove the endogenous synthesis of citric acid in the dog. This important question could now be decided by further experiments in which the citrate of the bones is taken into account. The same authors' preliminary experiments on the effect of alkalosis on citrate excretion in the rat, however, suggest that this point could be more easily proved by the use of this species [cf. Sober *et al.* 1940]. This would be very desirable in order to establish that the synthesis of citrate which Krebs & Eggleston [1940] observed *in vitro* also occurs in the living animal.

Summary

1. The citric acid content of the solid tissues of several species of animals has been determined.

2. It has been found that the hard substance of bone contains a hitherto unsuspected store of citric acid, which may constitute some 70% of that contained in the whole body. The marrow and cartilage contain much smaller amounts. Variations in the citrate content of bone, believed to be due to hor-

monal and dietary conditions, are reported. The variation of citrate greatly exceeds that of the other bone constituents, suggesting that the citrate is in a readily available form *in vivo*. *In vitro* it is not readily extracted from dried powdered bone by water, alcohol or ether.

3. Pure citric acid has been isolated from dried bone meal, and pure pentabromoacetone has been prepared from a trichloroacetic acid filtrate of bone meal in 80% of the yield calculated from analysis. It is concluded that the substance estimated in bone is in fact citric acid.

4. The skin and hair have high contents of citric acid, which may arise, at least in part, from the sebaceous secretion: a high content was found in a human sebaceous cyst. The citrate present in hair is to a large extent extractable by water.

5. The very young (3- to 5-day) chick embryo is rich in citric acid, and the amount present is sufficient to combine with one-third of the total embryonic calcium. As development proceeds, Ca increases more rapidly than citrate.

6. Tumour tissue usually, though not invariably, contains much citric acid.

7. The need for revision of certain experiments on the citric acid balance in animals, caused by the present findings, is pointed out.

8. It is concluded that the existing hypotheses of 'citrate-like substances' being concerned in calcium metabolism receive their main experimental support from the present detection of citrate itself as a major constituent of bone.

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117. INTERACTION OF ALDOSES WITH *α*-AMINO-ACIDS OR PEPTIDES

3. DETERMINATION OF EQUILIBRIUM CONSTANTS

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It has been shown in previous investigations [Frankel & Katchalsky, 1937; 1938] that the *p*H depressions occurring in mixtures of an aldose and an amino-acid or peptide in solution can be utilized to investigate the interaction between the amino and aldehyde groups. It is intended in the present paper to calculate the equilibrium constants of the system glucose + amino-acid or peptide from the measured *p*H depressions.

As pointed out previously [Frankel & Katchalsky, 1937], the reaction in question bears a close resemblance to the well investigated reaction between formaldehyde and amino-acids or peptides. It seemed probable that the reaction scheme developed for the formaldehyde titration [Levy, 1933; Balson & Lawson, 1936] would apply also to the reaction of aldoses with amino-acids or peptides. Measurements reported here fully confirmed this consideration.

The measurements were carried out potentiometrically on solution mixtures of different concentrations in the *p*H range 7–9. Within this *p*H range the reaction attains equilibrium in a relatively short time (3–48 hr.) and no interference of side reactions was observed.

The reaction could be described satisfactorily on the assumption that one molecule of glucose reacts with one molecule of amino-acid or peptide. In the range *p*H 7–9 a single constant is therefore sufficient to describe the equilibrium; at higher *p*H values, however, besides decomposition the occurrence of reactions of a higher order is not excluded. As according to the zwitterion theory only the anion of the amino-acid or peptide carries the unionized amino-group it is assumed that the interaction takes place between the anion of the amino-component and the undissociated glucose, and that the reaction product is completely dissociated and exists in solution only in the form of its anions.

Equilibrium constants of the following systems were determined: glucose and one of the following: glycine; glycylglycine; diglycylglycine; glycylalanine; glycylleucine; alanine; alanylglycine; alanylleucylglycine; leucine; leucylglycine; leucylglutamic acid; leucylphenylalanine; leucylglycylglycine.

On comparing the equilibrium constants of the reaction between glucose and various peptides it was found that the magnitude of the constant was mainly determined by the terminal amino-acid bearing the free amino group.

Theoretical

The reaction scheme used is similar to that of Levy [1933] and of Balson & Lawson [1936] for the formaldehyde titration. It is applied to the case of a bimolecular reaction and developed in the following way:

$$\begin{array}{ccc} & \overset{K}{\rightleftharpoons} & \\ HR & & R^- + H^+ \\ & + & \\ & G & \\ & \uparrow\downarrow L & \\ & RG^- & \end{array} \qquad K = \frac{H^+ . R^-}{HR}$$

$$L = \frac{RG^-}{R^- . G}$$

Where HR is the concentration of the zwitterionic form of the amino-acid or peptide ($^-$OOC—R—NH_3^+); R^- is the concentration of the anionic form ($^-$OOC—R—NH_2); G the total concentration of the glucose[1]; H the hydrogen ion concentration of the system; K the apparent acidic dissociation constant of the amino-acid or peptide, and L is the equilibrium constant of the reaction between glucose and the amino-acid or peptide.

Denoting the ionized part of the amino-acid or peptide by α, whether existing in the free state or combined with glucose, and the zwitterionic or undissociated part by $1-\alpha$, we obtain according to the reaction scheme:

$$\frac{\alpha}{1-\alpha}=\frac{R^-+RG^-}{HR}. \qquad \text{.....(1)}$$

Expressing the values of RG and HR by L, K, and R we obtain

$$\frac{\alpha}{1-\alpha}=\frac{R^-+L.R^-.G}{\frac{H}{K}R^-}=\frac{1+L.G}{\frac{H}{K}}, \qquad \text{.....(2)}$$

or

$$H=K\,(1+L.G)\,\frac{1-\alpha}{\alpha} \qquad \text{.....(3)}$$

introducing $p\mathrm{H}=-\log \mathrm{H}$ and $pK=-\log K$ we obtain

$$p\mathrm{H}=pK-\log\,(1+L.G)+\log\frac{\alpha}{1-\alpha}. \qquad \text{.....(4)}$$

The last equation shows that as long as the concentration of the glucose is kept constant the reaction scheme adopted leads to equations similar in form to that of Henderson as modified by Hasselbalch [1916]. The apparent dissociation constant of the system involves the equilibrium constant (L) and the dissociation constant of the amino-acid or peptide (K).

The pH of a solution of the component amino-acid or peptide of equal concentration and containing the same amount of alkali as the reaction mixture will be according to Henderson-Hasselbalch's equation

$$p\mathrm{H}'=pK+\log\frac{\alpha}{1-\alpha}. \qquad \text{.....(5)}$$

As the alkali concentrations both in the solution of the amino-acid or peptide and in the solution mixture with glucose are equal, it may be safely assumed that the degrees of ionization (α) in both systems are equal, i.e. $\frac{\alpha}{1-\alpha}$ appearing in equations (4) and (5) has the same value. From equations (4) and (5) we obtain calculated values for the pH depressions which can be verified experimentally.

$$p\mathrm{H}'-p\mathrm{H}=\Delta p\mathrm{H}=\log\,(1+L.G) \qquad \text{.....(6)}$$

denoting the antilogarithm of the pH depression by A we obtain

$$A=\text{antilog}\,\Delta p\mathrm{H}=1+L.G. \qquad \text{.....(7)}$$

Equation (7) provides an easy means of evaluating the equilibrium constants L from the known glucose concentrations and the measured pH depressions. The values of L obtained for different concentrations of glucose were sufficient to prove the validity of equation (7).

[1] Two simplifying assumptions, which seemed to be reasonable under the experimental conditions used, were made. (1) The glucose was considered as an ideal non-electrolyte in the pH range of the experiments. (2) Since the concentration of glucose was many times higher than that of the amino-component and RG was negligible in comparison with G, the concentration of *free* glucose (G) was considered equal to the *total* concentration of glucose.

Results

Table 1 illustrates the use of equation (7) for the calculation of the equilibrium constant in the reaction of glucose with diglycylglycine. The calculation for the other cases was carried out in a similar manner.

Table 1. *Interaction of diglycylglycine with glucose*

Initial *p*H 7·09. 23°

G Conc. of glucose *M*	*p*H at equilibrium	Δ*p*H	A Antilog Δ*p*H	*L* Equilibrium const.
0·1	6·61	0·48	3·02	20·2
0·3	6·25	0·84	6·92	19·7
0·5	6·05	1·04	10·96	19·9
0·7	5·90	1·19	15·49	20·7
0·9	5·80	1·29	19·50	20·5

$L = 20{\cdot}2$.

The equilibrium constants of the systems glucose + various amino-acids or peptides are listed in Table 2..

Table 2. *Equilibrium constants of the reaction of glucose with amino-acids or peptides*

Glycine and glycyl-peptides		Alanine and alanyl-peptides		Leucine and leucyl-peptides	
Substance	*L*	Substance	*L*	Substance	*L*
Glycine	8·6	Alanine	6·7	Leucine	4·2
Glycylglycine	20·8				
Glycylalanine	17·3	Alanylglycine	7·4		
Glycylleucine	21·8			Leucylglycine	5·8
Diglycylglycine	20·2	Alanylleucylglycine	8·6	Leucylglycylglycine	5·9

Somewhat remarkable are the high values of the equilibrium constants of glycine and glycyl-peptides. Table 2 indicates that as might be expected in the simple aliphatic peptides the equilibrium constant of the reaction with glucose, and correspondingly the *p*H depressions, are mainly determined by the terminal amino-acid bearing the free amino-group. Thus the differences in the *p*H depressions even measured colorimetrically [Frankel & Katchalsky, 1937] constitute a method of determining the terminal amino-acid in a simple peptide.

Some peptides containing an aromatic group (phenylalanine) or an additional carboxyl group do not conform to the above regularity as shown in Table 3.

Table 3

Substance	*L*
Leucylglycine	5·8
Leucylglutamic acid	7·3
Leucylphenylalanine	8·4

Experimental

The determination of the *p*H depressions as a function of the glucose concentration was carried out as follows. Two sterile stock solutions, one of amino-acid or peptide and one of glucose, considerably more concentrated, were prepared. The stock solution of the amino component was brought to the desired *p*H by suitable additions of NaOH. To the glucose no alkali additions were necessary as glucose is practically a non-electrolyte and therefore has no effect

on the *p*H. The necessary volumes of the stock solutions to give mixtures of the compositions amino-acid or peptide solution to glucose solution of 1 : 9, 3 : 7, 5 : 5, 7 : 3, 9 : 1, were withdrawn and mixed. As the amino-acid and peptide solutions are fairly strong buffers in the *p*H range used, the dilution by mixing had no appreciable effect on the initial *p*H. Thus a series of isohydric mixed solutions of different glucose concentrations was obtained. The mixed solutions were kept in sterile test tubes at 23° for 5–6 days. Samples of 0·5–1 ml. were withdrawn under sterile conditions from each solution and the *p*H determined by means of the hydrogen electrode. The first measurements were performed immediately after mixing and were continued for 3 hr., as during the first hours the *p*H changes were rapid and large. In general the values became constant after 25–48 hr., nevertheless the measurements were continued for 5–6 days.

A constant potential at the hydrogen electrode was achieved after 2–5 min. with 0·5–1 ml. of solution. The measurements made for several days after equilibrium was reached agreed within 1–2 mV. All the calculated constants are therefore liable to deviations within this range of error.

The glucose used throughout was 'glucose purissimum' of Merck. All the amino-acids were products of Hoffman-la-Roche; most peptides were prepared in this laboratory and the remainder obtained from la-Roche. All the products were recrystallized and their purity determined by analysis. The stock solutions were freed from micro-organisms by filtering through a purified Seitz filter and kept in tightly stoppered, sterile test tubes. During the withdrawal of samples precautions were taken to secure sterility.

Summary

1. The equilibrium constant of the interaction of glucose with amino-acids or peptides was calculated. The underlying assumption is that the reaction is bimolecular and proceeds between glucose and the anion of the amino component.

2. The values of the equilibrium constants obtained from potentiometric measurements within the *p*H range 7–9, show a satisfactory agreement for various concentrations.

3. In the case of peptides the magnitude of the equilibrium constants was found to be mainly determined by the terminal amino-acid of the peptide bearing the free amino-group.

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118. INTERACTION OF ALDOSES WITH α-AMINO-ACIDS OR PEPTIDES

4. THE PERCENTAGE COMBINATION

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In the previous papers of this series [Frankel & Katchalsky, 1937; 1938] evidence was put forward for the interaction of amino-acids and peptides with aldoses and for the concept that the *p*H depressions serve as a measure of the interaction. It has further been shown that at equilibrium the *p*H depressions are quantitatively related to the equilibrium constants of the reaction [Katchalsky, 1941]. But the *p*H depressions do not directly express the amount of the reaction product formed.

Previous authors have tried to measure the concentration of the reaction product by various methods, either during the reaction course [Borsook & Wasteneys, 1925; Waldschmidt-Leitz & Rauchalles, 1928; Shiga, 1937] or at equilibrium [Neuberg & Kobel, 1925; 1928; Euler & Brunius, 1928]. Their results are to some extent contradictory and cannot be combined to give a uniform picture.

In the present paper it will be shown that the amount of the reaction product formed can be calculated from the *p*H depressions during the reaction as well as at equilibrium. Furthermore, it is shown that the experimental data for the amount of combination obtained by the measurement of the diminution of free NH_2-N of the system are in a very satisfactory agreement with the results derived from potentiometric measurements.

Theoretical

Let us consider the reaction from its start to time t. For the initial hydrogen ion concentration the Henderson formula holds:

$$H_0 = K\frac{1-\alpha}{\alpha}, \qquad \text{.....(1)}$$

where H_0 denotes the initial hydrogen ion concentration; K denotes the apparent acidic dissociation constant of the amino-acid or peptide, and α denotes the degree of ionization of the amino-component.

At time t the hydrogen ion concentration of the system has increased to H_t and the *p*H has decreased to the value pH_t, the *p*H depression being represented by $\Delta pH_t = pH_0 - pH_t$. A fraction of the anions of the amino-acid or peptide (R^-) has evidently combined with the glucose to give the reaction product RG^-. At time t the ratio of the ionized to the unionized part of the amino-acid or peptide is given by the expression

$$\frac{R_t^- + RG_t^-}{HR_t} = \frac{\alpha}{1-\alpha}. \qquad \text{.....(2)}$$

It can safely be assumed that the reaction between glucose and the non-ionic amino group proceeds much more slowly than the attainment of equilibrium in

the ionic system $H_t^+ + R_t^- = HR_t$. The following equation (3) is therefore valid for any time t

$$\frac{H_t . R_t^-}{HR_t} = K. \qquad \text{.....(3)}$$

On introducing the value of HR_t from equation (3) in equation (2) we obtain

$$\frac{R_t^- + RG_t^-}{\frac{H_t . R_t}{K}} = \frac{\alpha}{1-\alpha},$$

or

$$H_t = K \frac{1-\alpha}{\alpha} \frac{R_t^- + RG_t^-}{R_t}. \qquad \text{.....(4)}$$

As the alkali concentration of the system does not change during the interaction, the concentration of the anions remains constant. Thus for a given pH, $\frac{1-\alpha}{\alpha}$ does not change during the interaction and can therefore be introduced into equation (4) from equation (1).

$$H_t = H_0 \frac{R_t^- + RG_t^-}{R_t}, \qquad \text{.....(5)}$$

$$\therefore \quad \frac{RG_t^-}{R_t} = \frac{H_t}{H_0} - 1.$$

$\frac{H_t}{H_0}$ is the antilogarithm of the pH depression at time t, i.e.

$$\frac{H_t}{H_0} = \text{antilog}\, \Delta pH_t = A_t.^1$$

Hence

$$\frac{RG_t^-}{R_t} = A_t - 1 \quad \text{or} \quad R_t^- = \frac{RG_t^-}{A_t - 1}. \qquad \text{.....(6)}$$

The known concentration of the amino-acid or peptide (c) is the sum of the concentrations of the undissociated molecules, of the anions and of the compound with glucose, i.e.

$$c = HR_t + R_t^- + RG_t^-. \qquad \text{.....(7)}$$

Introducing the value of HR_t from equation (3) and of R_t^- from equation (6) we obtain

$$c = RG_t^- \left[\frac{1}{A_t - 1} \left(1 + \frac{H_t}{K} \right) + 1 \right], \qquad \text{.....(8)}$$

or

$$\frac{RG_t^-}{c} = \frac{K\,(A_t - 1)}{K . A_t + H_t}.$$

As $H_t = H_0 . A_t$, equation (8) can be transformed into

$$\frac{RG_t^-}{c} = \frac{K}{K + H_0} \frac{A_t - 1}{A_t}. \qquad \text{.....(9)}$$

$100 \frac{RG_t^-}{c}$ is the percentage (p_t) of the amino-acid or peptide combined with the glucose at time t.

$$100 \frac{RG_t^-}{c} = p_t = 100 \frac{K}{K + H_0} \frac{A_t - 1}{A_t}. \qquad \text{.....(10)}$$

From equation (10) it is possible to calculate the amount of the reaction product at any time if the pH depression, the dissociation constant of the amino-acid or peptide and the initial pH are known.

At equilibrium A_t becomes the equilibrium value A which was shown [Katchalsky, 1941] to be $1 + L.G$ (L denoting the equilibrium constant of

[1] $\Delta pH_t = pH_0 - pH_t = -\log H_0 + \log H_t = \log \frac{H_t}{H_0}$.

the reaction in question; G the concentration of glucose). Introducing the last expression into equation (10) we obtain the percentage of the amino-acid or peptide combined with glucose at equilibrium:

$$p = \frac{K}{K+H_0}\,\frac{L.G}{1+L.G}\,100. \qquad \text{.....(11)}$$

The percentage of the reaction product present at equilibrium reaches a maximum at high initial pH when $H_0 \ll K$. The maximum can be deduced from equation (11) on neglecting H_0 in comparison with K

$$p_{max.} = 100\,\frac{L.G}{1+L.G}. \qquad \text{.....(12)}$$

Results

We checked our formulae deriving from the pH depressions the percentage combination of the amino-component with glucose by determining this amount by another independent method, i.e. estimation of the free amino groups by Van Slyke's volumetric method. The results of Shiga [1937] and others show that Van Slyke's method is suitable for our purpose. Previous workers have always worked in the presence of a buffer. Since the percentage combination of the amino-component depends on the pH, the addition of a buffer would affect considerably the results obtained. Accordingly all our experiments were made without the addition of any external buffer.

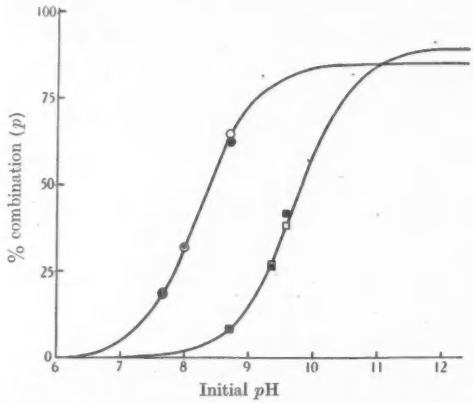

Fig. 1. Percentage combination at equilibrium of glycine or leucylglycine with glucose for various initial pH values.

- o glycine (0·1 M) + glucose (M) calc. from potentiometric data.
- • glycine (0·1 M) + glucose (M) measured by NH_2-N diminution.
- □ leucylglycine (0·05 M) + glucose (M) calc. from potentiometric data.
- ■ leucylglycine (0·05 M) + glucose (M) measured by NH_2-N diminution.

Fig. 1 represents the percentage combination of glycine and leucylglycine with glucose at equilibrium for different initial pH. The experimental values of the NH_2-depressions determined gasometrically were found to be on the curves calculated from the potentiometric data. The curves show that in order to obtain a fixed percentage of combination a higher pH is required in the case of the amino-acid than in that of the peptide. This is due to the difference in the

magnitudes of the apparent acidic dissociation constants (cf. equation (11)) of glycine ($K=2\times10^{-10}$) and leucylglycine ($K=6\times10^{-9}$). The different 'reactibilities' of amino-acids and peptides, discussed already in the earliest papers dealing with the reaction in question, thus appear to be mainly due to the difference in the well-known dissociation constants.

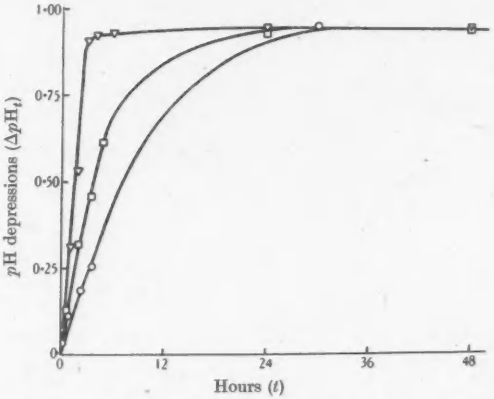

Fig. 2. Course of pH depressions with time in the interaction of glycine (0·1 M) with glucose (M) for different initial pH values.

▽ Initial pH = 8·71. □ Initial pH = 9·34. ○ Initial pH = 9·57.

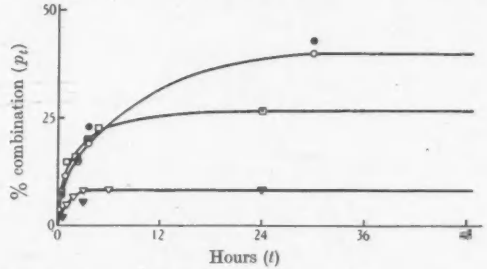

Fig. 3. Increase of percentage combination with time in the interaction of glycine (0·1 M) with glucose (M) for different initial pH values.

Initial pH 8·71; ▽ calc. from potentiometric data; ▼ measured by NH_2-N decrease.
Initial pH 9·34; □ calc. from potentiometric data; ■ measured by NH_2-N decrease.
Initial pH 9·57; ○ calc. from potentiometric data; ● measured by NH_2-N decrease.

As indicated by equation (11) and verified experimentally the percentage combination is small at pH values lower than the isoelectric point of the amino-components, increases with increasing pH and attains maximal values at high alkalinities. These maximal values are independent of the dissociation constants (K) and thus show distinctly the differences in the equilibrium constants (L). The maximal values could not, however, be achieved without introducing large errors due to the decomposition of glucose discussed in previous papers.

In Figs. 2–5 the course of the interaction is followed by both experimental methods. Figs. 2 and 4 show the course of the pH depressions with time for

different initial pH values, while Figs. 3 and 5 show the corresponding increase in the percentage of the reaction product calculated from the potentiometric data and measured by Van Slyke's gasometric method. The agreement between the results of both methods is good throughout.

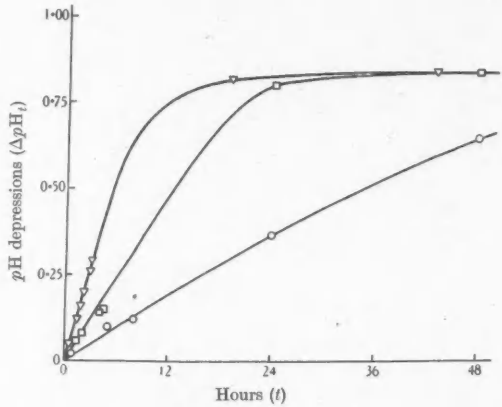

Fig. 4. Course of pH depressions with time in the interaction of leucylglycine (0·05 M) with glucose (M) for different initial pH values.

▽ Initial pH = 7·66. □ Initial pH = 7·99. ○ Initial pH = 8·70.

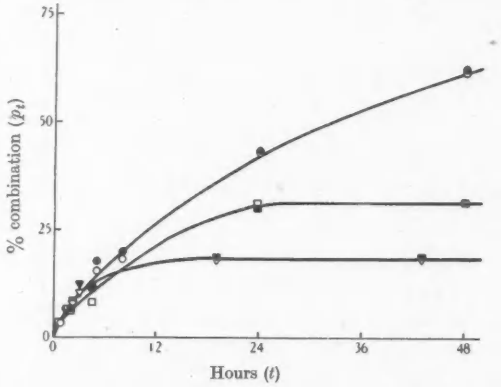

Fig. 5. Increase of percentage combination with time in the interaction of leucylglycine (0·05 M) with glucose (M) for different initial pH values.

Initial pH 7·66; ▽ calc. from potentiometric data; ▼ measured by NH_2-N decrease.
Initial pH 7·99; □ calc. from potentiometric data; ■ measured by NH_2-N decrease.
Initial pH 8·70; ○ calc. from potentiometric data; ● measured by NH_2-N decrease.

The pH depressions in the reaction of glycine or leucylglycine with glucose reach a constant value dependent only upon the equilibrium constant of the reaction system (cf. Katchalsky [1941], $\Delta pH = \log(1 + L.G)$), although the time necessary for the achievement of equilibrium increases with increasing

initial *p*H. The percentage-time curves show on the other hand different final values, increasing with increasing initial *p*H, corresponding to the equilibrium data of Fig. 1.

We hope to discuss the kinetics of the interaction in more detail in a future paper.

Experimental

The potentiometric measurements were carried out exactly as described in the previous paper [Katchalsky, 1941].

The gasometric NH_2-N measurements were carried out in the micro Van Slyke apparatus for volumetric determinations. The reagents were those recommended by Kendrick & Hanke [1937]; Dunn & Porush [1939] have remarked that in the presence of iodine glucose would not interfere with the precision of the results.

All the materials used were twice recrystallized and analysed before use. Sterility was maintained during the preparation of the solutions as well as during the withdrawal of samples for the *p*H and NH_2-N determinations.

SUMMARY

1. It is shown that the amount of the reaction product formed from glucose and amino-acids or peptides can be calculated from the actual *p*H depressions.

2. The values calculated from potentiometric measurements were verified by measurements of NH_2-N diminution during the course of the reaction as well as at equilibrium.

3. The percentages of combination at equilibrium are small at *p*H values lower than the isoelectric point of the amino components, increase with increasing *p*H and attain maximal values at high alkalinities.

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119. INTERACTION OF ALDOSES WITH *a*-AMINO-ACIDS OR PEPTIDES

5. CONTINUOUS BACK TITRATION OF THE SYSTEM TO ITS INITIAL *p*H

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Shiga [1938] re-examined the change in *p*H resulting from the combination of amino-acids or peptides with glucose by the method reported in the first paper of this series [Frankel & Katchalsky, 1937]. He confirms our experimental results but claims that 'the displacement in *p*H does not necessarily indicate the amount of acid produced and is greatly influenced by the buffering capacity of the solution'. Shiga comes to the conclusion that it is not the *p*H depression but the amount of alkali necessary to restore the *p*H to its initial value that represents the amount of combination. He found that in the back titration of glycylglycine and glucose at various *p*H values the required additions of alkali reached a maximum near *p*H 8, while no such maximum was detected in the case of glycine and glucose; he therefore came to the conclusion that the interactions in the two cases are of different types.

In the preceding paper [Frankel & Katchalsky, 1941] the amount of combination was calculated and verified experimentally. In this paper it will be shown that Shiga's interpretation of the results obtained in his back titration method is erroneous. A continuous back titration method has been used, in which the *p*H of the system is kept constant during the interaction by suitable additions of alkali till equilibrium is attained. The relation between the percentage combination of the NH_2-bearing component with glucose and the total alkali added at any moment was considered. The amount of alkali added when equilibrium was reached was calculated from the *p*H of the particular experiment and the constants: K, the apparent acidic dissociation constant of the amino-component, and L, the equilibrium constant of the reaction system, as determined by Katchalsky [1941]. The agreement between the experimental and calculated values was very satisfactory throughout.

This quantitative treatment is supported by new experimental data.

Theoretical

Let us assume that the alkali contained in the reaction mixture of amino-acid or peptide with glucose at the start of the reaction is b, and the concentrations of the amino component and of glucose are c and G respectively. As glucose can be considered a non-electrolyte, the alkali concentration determines the hydrogen ion concentration of the system according to the equation

$$\mathrm{H} = K\frac{c-b}{b}, \qquad \text{.....(1)}$$

or

$$b = \frac{K.c}{K+\mathrm{H}}. \qquad \text{.....(2)}$$

After a time interval t, anions of the complex RG^- (of conc. RG_t^-) are formed and in order to maintain the pH at its initial value an amount of alkali Δb_t is to be added, such that the total alkali concentration is then given by $b+\Delta b_t$. The total concentration of the amino-acid or peptide is given by[1]

$$c = HR_t + R_t^- + RG_t^-, \qquad \text{.....(3)}$$

$$\therefore \quad c - RG_t = HR_t + R_t.$$

Assuming that equilibrium between the ions of the amino component is attained instantaneously, i.e. that $\frac{H.R_t^-}{HR_t} = K$ is valid throughout the interaction, we obtain

$$R_t^- = \frac{K(c - RG_t^-)}{K+H}. \qquad \text{.....(4)}$$

The condition of electroneutrality requires that

$$b + \Delta b_t = R_t^- + RG_t^-. \qquad \text{.....(5)}$$

Introducing the value of b from equation (2) and of R_t^- from equation (4) we obtain

$$\frac{K.c}{K+H} + \Delta b_t = \frac{K(c - RG_t^-)}{K+H} + RG_t^- = \frac{K.c}{K+H} + RG_t^-\left(1 - \frac{K}{K+H}\right), \qquad \text{.....(6)}$$

$$\Delta b_t = RG_t^- \frac{H}{K+H}. \qquad \text{.....(7)}$$

Equation (7) represents the required expression for the relation between the amount of the complex present and the total alkali addition at any time during the reaction. It shows that the amount of alkali required depends not only on the amount of complex present but also on the hydrogen ion concentration of the system.

In the previous paper the expression $100\,\frac{RG^-}{c}$ was defined as the percentage combination of the glucose with the amino-acid or peptide; this percentage is readily derived from the last expression and thus can be measured by the continuous back titration method.

$$p_t = 100\,\frac{RG_t^-}{c} = 100\,\frac{\Delta b_t}{c}\,\frac{K+H}{H}. \qquad \text{.....(8)}$$

At equilibrium the percentage combination is represented by equation (8) of the previous paper, taking into account that H is the hydrogen ion concentration after equilibrium had been reached (as distinct from the initial hydrogen ion concentration appearing in equations (9) and (10) of that paper), i.e.

$$p = 100\,\frac{K(A-1)}{H + K.A} = 100\,\frac{K.L.G}{H + K(1 + L.G)},$$

comparing the last expression with equation (8) of the present paper we obtain the amount of alkali to be added until equilibrium, expressed by the dissociation constant of the amino-acid or peptide, by the equilibrium constant of the reaction system and the concentration of glucose

$$\frac{\Delta b}{c} = \frac{K.L.G}{H + K(1 + L.G)}\,\frac{H}{K+H}. \qquad \text{.....(9)}$$

[1] HR_t, R_t^- and RG_t^- represent the concentrations of the unionized (or zwitterionic) molecules of the amino-acid or peptide, the free anions of the amino-component and those combined with glucose respectively, at time t.

The curve representing $\frac{\Delta b}{c}$ as a function of pH (equation (9) has a maximum. The maximum value of the alkali additions is found by differentiation to be given by

$$\left(\frac{\Delta b}{c}\right)_{\text{max.}} = \frac{\sqrt{A}-1}{\sqrt{A}+1}. \qquad \text{.....(10)}$$

The corresponding pH being

$$p\text{H}_{\text{max.}} = p\text{K} - \tfrac{1}{2}\log A = p\text{K} - \tfrac{1}{2}\log(1+L.\text{G}). \qquad \text{.....(11)}$$

Results

The experimental verification of the equations deduced above was carried out as follows: solutions of glucose to which suitable indicators were added, were

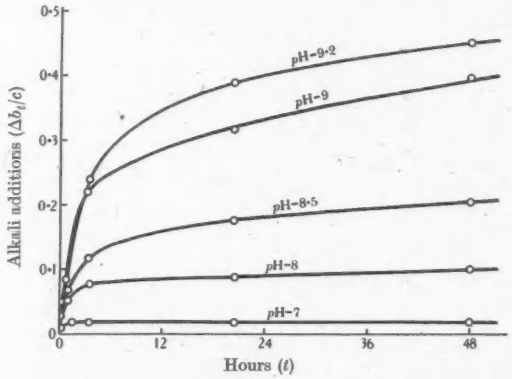

Fig. 1. Additions of alkali ($\Delta b_t/c$) necessary to keep the solution mixtures at various initial pH values during the interaction of glycine (0·1 M) with glucose (M).

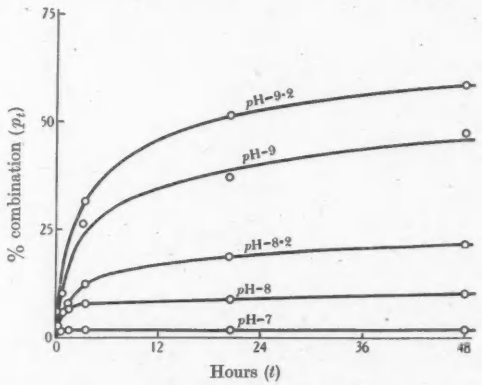

Fig. 2. Increase of percentage combination in the interaction between glycine (0·1 M) and glucose (M) for various initial pH values which are kept constant.

brought to pH 7, 8, 8·5, 9, 9·2, and 9·7 by the addition of alkali and diluted until the concentration was 2 M; similarly solutions of glycine were brought to the

same pH values and diluted until the concentration was 0·2 M. 5 ml. of each glucose solution were withdrawn and mixed with 5 ml. of the glycine solution of equal pH. Thus solutions of different pH but having the same glucose concentration (M) and the same glycine concentration (0·1 M) were obtained. The pH values, as measured colorimetrically, started to decrease with different velocities and suitable amounts of alkali were added from time to time throughout the course of the reaction to maintain the pH values at their initial levels. The values of $\frac{\Delta b_t}{c}$ obtained from the total amounts of alkali added and the calculated p_t values are represented in Figs. 1 and 2 respectively.

The colorimetric data indicate that the velocity constant was approximately proportional to the concentration of the hydrogen ions in the solution; or that the reaction velocity decreases with increasing pH.

The equilibrium data calculated according to equation (9), as well as the measured values, are represented in Fig. 3. The agreement of the experimental

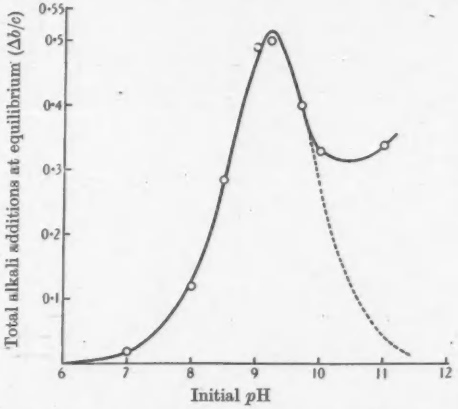

Fig. 3. Total alkali additions necessary to keep the mixture solutions of glycine (0·1 M) and glucose (M) at various initial pH values until equilibrium is reached.

values with the calculated curve is very good throughout. At very high alkalinities the quantity of alkali actually added was higher than the calculated value. This fact is explained by the decomposition of glucose already discussed in previous papers [cf. Frankel & Katchalsky, 1937].

A closer examination of Fig. 3 reveals the cause of the apparent difference in the 'reaction types' of the peptides and the amino-acids, described by Shiga [1938]. While the pH of the maximum alkali addition lies in the case of the peptides near pH 7·5–8 (e.g. glycylglycine: pK = 8·2; L = 20·8; G = 1; $p\mathrm{H}_{\mathrm{max.}} = p\mathrm{K} - \frac{1}{2}\log(1 + L.\mathrm{G}) = 8{\cdot}2 - \frac{1}{2}\log 21{\cdot}8 = \mathbf{7{\cdot}53}$) it lies in the case of amino-acids near pH 9 (e.g. glycine: pK = 9·7; L = 8·6; G = 1; $p\mathrm{H}_{\mathrm{max.}} = 9{\cdot}7 - \frac{1}{2}\log(1 + 8{\cdot}6) = \mathbf{9{\cdot}2}$). Shiga carried out his experiments only up to pH 9 and while observing the maximum of the NaOH additions in the case of peptides he failed to reveal it in the case of amino-acids and believed the curve to increase steadily with increasing pH. Our measurements were extended over a larger pH range and the maximum appears in the case of glycine as distinctly as in the case of glycylglycine.

SUMMARY

1. It is shown that contrary to the interpretation of Shiga [1938] the alkali additions necessary to keep the *p*H of a mixture of solutions of glucose and glycine at its initial value, do not express directly the amount of reaction product.

2. The percentage combination was calculated from the total alkali added at any moment during the reaction and the hydrogen ion concentration of the system. The reaction velocity decreases with increasing *p*H.

3. The total alkali additions for various *p*H values, when equilibrium is reached, show a maximum.

4. Glycine requires a maximum of alkali additions near *p*H 9, while glycylglycine shows the maximum at *p*H 7·5–8. This fact is explained by the differences in the acidic dissociation constants of glycine and glycylglycine and by the different equilibrium constants of their interaction with glucose (cf. equation (11)). Shiga's view that the reactions of glycine and glycylglycine with glucose are of different types is therefore erroneous.

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120. VARIATIONS IN CERTAIN PROPERTIES OF BOVINE CERVICAL MUCUS DURING THE OESTROUS CYCLE

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THE epithelium of the cervix and upper vagina of the cow and certain other animals produces a mucous secretion in which, according to Woodman & Hammond [1925], may be found a mucoprotein belonging to the class of mucin bodies containing mucoitin sulphuric acid, since they identified glucosamine in a specimen of cervical mucus. The chemical composition of this secretion does not appear to have been further investigated since this observation was made, but the scanty evidence available indicates that it may contain a mucopolysaccharide-protein complex (see Meyer's [1938] classification of mucopolysaccharides and glycoproteins).

Changes in the amount and physical properties of bovine cervical mucus were described by Woodman & Hammond [1925], who pointed out that at oestrus the secretion is plentiful, fairly fluid and capable of being drawn out into threads, whereas after oestrus it is considerably diminished in amount and less fluid. The amount of secretion, however, differs markedly in different animals and cannot be taken as a definite indication of oestrus. In pregnancy the secretion is very thick and adhesive, effectively sealing the gravid uterus, and Marshall & Hammond [1937] have described a rough, subjective test for pregnancy in the cow based on recognition of the characteristic stickiness of the cervical secretion. The work described in this paper forms part of a study of the chemical and rheological properties of bovine cervical mucus in different phases of the reproductive cycle, undertaken with a view to attempting to develop the mucin test of Marshall & Hammond [1937] into an objective test for early pregnancy in the cow.

As a first step it was considered necessary to study the changes in the properties of the secretion during the oestrous cycle in order to provide a standard with which to compare the behaviour of these properties during pregnancy. As has been shortly described in a preliminary note [Scott Blair *et al.* 1941], these studies have led to the development of a simple and rapid rheological test for oestrus or near-oestrus which may prove useful in the diagnosis of bovine oestrus in cases, and these are not infrequent [see Hammond, 1927], in which the external signs are not obvious even to a skilled herdsman, and also for predicting its imminence when, as in cold winter weather, it is to be expected that it may be easily missed on account of its short duration, particularly when it occurs at night.

Such studies might also be significant in connexion with the physiology of the passage of sperm into the uterus after coitus and might throw light on conditions which allow of bacterial invasion of the uterus through the cervix with consequent sterility.

The properties which have been included in this study are as follows:
(1) Chemical properties (*a*) total nitrogen, (*b*) water content.
(2) Rheological properties (*a*) viscosity, (*b*) flow-elasticity.

Experimental

Mucus samples

Samples were taken by the insertion of the hand into the vagina. The vulva was washed with a warm dilute solution of 'Dettol' and after one hand had been washed, disinfected and dried, liquid paraffin was applied to its back leaving the underside quite dry. Thus lubricated, the hand could be gently inserted into the vagina, extreme care being used and the line of least resistance always taken. It proved to be impracticable to obtain sufficient mucus for our purpose from the entrance to the cervix, so that the technique adopted as standard was to take as much mucus as possible from the vagina in the immediate vicinity of the cervix.

Throughout the work there was no indication that repeated sampling, twice a week as a rule and sometimes as often as three times weekly, led to any damage or infection of the reproductive tract likely to cause any change in the composition or properties of the cervical secretion or to hinder conception or disturb established pregnancy. For example, samples were taken from one of our cows at least once and usually twice a week for 10 weeks; she was then served, conceived to the first service and pregnancy proceeded normally despite the continued taking of mucus samples for a further 75 days, when she was slaughtered. It is inadvisable, and usually anatomically impossible, to take mucus samples by hand from the vaginae of nulliparous heifers.

Chemical analysis of secretions

Up to the present, chemical analyses have been confined to determinations of total N (micro-Kjeldahl) and total solids (by evaporation at 100°). Further studies are being made of the chemical properties of the secretions. Fig. 1 shows typical curves for individual cows which were sampled at regular intervals during one or two consecutive oestrous cycles and in one case well into pregnancy. The occurrence of oestrus as observed by the herdsman is indicated, and it will be seen that a sharp minimum in the total N content of the secretion occurs at about the time of oestrus, the N content falling to approximately one-tenth of the mid-cycle value. One cow, Clara 9, was served after samples had been taken over 2 cycles. Conception took place, as was known for certain when she was slaughtered 75 days later, and the periodicity in the total N content of the secretion immediately ceased, the values remaining at the relatively high level characteristic of mid-cycle samples. These observations indicate that a determination of the total N content of the secretion might be made the basis of a practical method for diagnosing oestrus, but, as will be seen, a far more rapid and simple test based on flow-elasticity was successfully devised so that these observations were not further extended.

Values for the total solids content of the samples from the same cows show a similar periodicity. As was to be expected from naked-eye observations of its physical properties, the water content of the secretion is highest at or near oestrus. This raises the question as to whether or not the fall in total N at oestrus is due merely to increase in water content. Calculated values (Fig. 1) for the total N content of the solid matter of the secretion indicate that this is not so.

The N content of the dry matter is minimal at oestrus with a value of 1–2%, while in mid-cycle it rises to a value of 6–10%. It may therefore be concluded that after oestrus nitrogenous substances enter the secretion. These are probably proteins arising from the breakdown of cellular débris from the epithelium of the reproductive tract, leucocytes, etc.

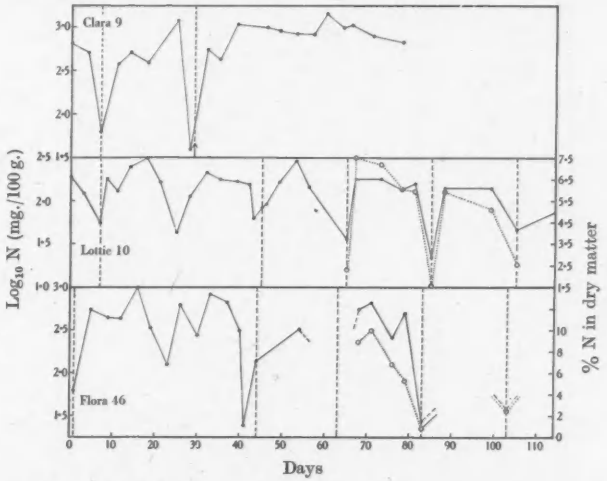

Fig. 1. Variations in total N of bovine cervical mucus during the oestrous cycle. Vertical broken line indicates observed oestrus and arrow denotes service. Dotted curves give percentage total N in dry matter (calculated).

Physical properties of secretions

(*a*) *Viscosity*

These secretions are by no means true fluids, that is to say, the viscosity is not independent of variations in stress and strain conditions. If a true fluid is caused to flow through a narrow tube, the amount of flow per second is proportional to the applied pressure. With the secretions, however, flow is relatively faster for high pressures than for low, and also the act of streaming itself causes a marked slowing down of flow for any given pressure (work-hardening). If it can be arranged, however, that the effective pressure on the flowing secretion increases at such a rate as approximately to offset the rise in viscosity due to the ever-increasing strain (the streaming), a viscosity can be obtained which, although by no means a constant of the material, should be sufficiently reproducible to be of value, especially since the viscosities of the secretions vary over a very wide range (sometimes of the order of 100,000 : 1) during the oestrous cycle.

Some years ago, a somewhat similar problem with honey led to the designing of a special viscosimeter [Scott Blair, 1937] in which a narrow glass tube is filled with the material to be investigated and is then emptied by means of a constant air pressure (P)[1] applied at one end. If the tube has a radius R and length L, and if the length of the column of secretion (which varies from L to O during the

[1] P must be quoted in dynes/cm.^{2}

emptying) is described as l for any given time t, it is easy to show that for a true fluid, curves plotting $Pt/(L^2-l^2)$ are linear and pass through the origin, and that their slope has only to be multiplied by $R^2/4$ to give the viscosity (η) in absolute units (poises).[1]

Since the applied pressure at the end of the tube is kept constant, the stress which is actually shearing the column of secretion along the wall of the tube must be increasing rapidly (the area of effective wall being $2\pi Rl$). The secretions do not flow in the streamline manner of a true fluid, but, as is shown in Fig. 2,

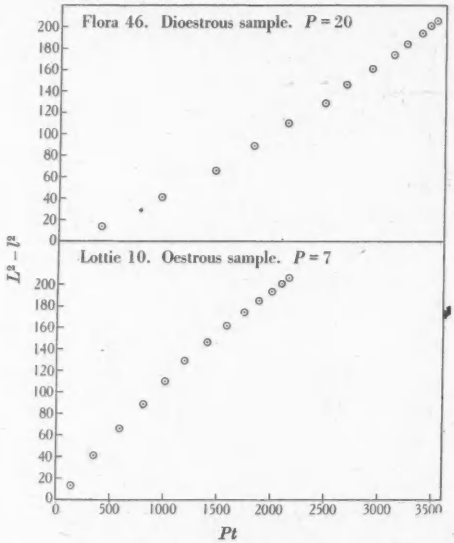

Fig. 2. Flow-curves for oestrous and mid-cycle samples of bovine cervical secretions in Scott Blair viscosimeter.

the curves are remarkably linear, hence the fall in viscosity produced by this rising stress (which can be independently shown by comparing viscosities taken at two different air-pressures) must be very closely offset by the work-hardening effect. The secretion toughens as it is stretched along the tube. Oestrous samples show some tendency to downward curvature, since in them work-hardening is enormous; mid-cycle samples tend rather the other way, but there is rarely any difficulty in drawing a fair straight line through the points. Sometimes a slight zero error introduces a small intercept on the Pt axis, but the curve remains very nearly linear.

Viscosities determined in this way on secretions roughly homogenized by squeezing between two glass plates vary from about a poise in oestrus to about 1000 poises in mid-cycle, though much wider variations than this often occur. Remembering that the viscosity of water at room temperature is about 0·01 poises (1 centipoise) the range of viscosities is clearly visualized, though in view of the large numbers involved we prefer to plot $\log_{10}\eta$ in studying variations

[1] Small corrections in this formula are discussed in the original paper but may be neglected in the present work.

during the oestrous cycle. Fortunately the temperature coefficient of η is remarkably small and no temperature control is required.

Fig. 3 shows typical curves for individual cows during several cycles, and in one case well into pregnancy; the dotted lines indicate oestrus as observed by the herdsman (often with warning from the laboratory).

In obtaining these curves, it was not always possible to use the same pressure for all the samples on account of the wide variations in viscosity. Whenever possible, measurements were made at a pressure of 40 cm. of water; when this was not feasible, measurements were made at two different pressures, the viscosities at the standard pressure of 40 cm. of water being determined by

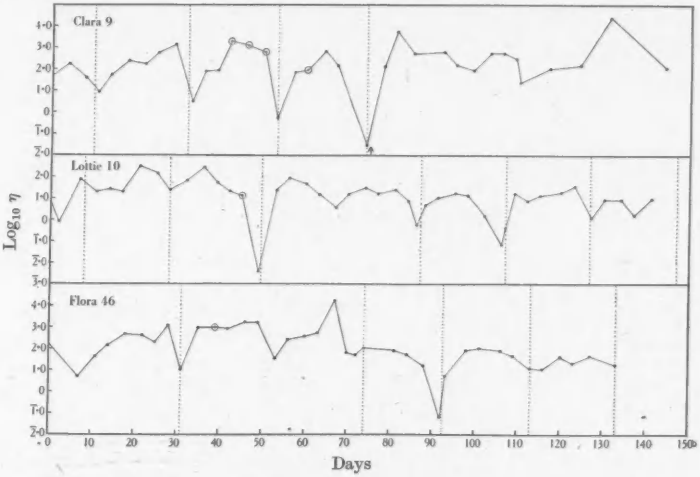

Fig. 3. Variations in viscosity of bovine cervical mucus during the oestrous cycle. Vertical broken line indicates observed oestrus and arrow denotes service. Points marked with a circle are somewhat unreliable due to errors in extrapolation at high viscosities.

extrapolation on log η/log P diagrams, which are fortunately fairly linear. There is a tendency for these log η/log P curves to be steeper in the case of oestrous samples. Viscosities determined in this way give a very fair indication of when a cow is in season or within a few hours either way of being in season, but the method is not advocated for practical purposes for the following reasons:

(1) The apparatus is too delicate and complicated for cowshed use and the test takes some time (at least 20 min. with calculation). It requires a certain amount of manipulative skill.

(2) On occasions, the secretions are apt to show misleadingly low viscosities when the cow is not in season. This is usually due to contamination of the mucus with urine because of urination before or during sampling, but occasionally it must be assumed to be due to other causes, such as catarrh associated with infection of the vagina. Samples contaminated with urine are generally detectable by appearance and smell.

(3) A much simpler and more reliable test is available, based on the measurement of 'flow-elasticity'.

(*b*) *Flow-elasticity*

If, during the extrusion of the column of secretion in the viscosimeter the pressure is suddenly released, the column recoils and flows back along the tube. For samples taken from cows in mid-cycle this recoil amounts to anything from 0 to about 4 mm. In animals actually in season, recoils are usually between 20 and 30 mm.; accuracy is low when recoil is much over 30 mm. This type of elastic recoil has been observed in other materials and discussed by earlier workers and was named by Szegvari 'Fliesselastizität', which is generally translated 'flow-elasticity' [Szegvari, 1924; Ostwald *et al.* 1933].

Table 1. *Effect of various factors on flow-elasticity* (ρ)

(*a*) Effect of varying the radius of the capillary on ρ

Tube no.	1	2	3	4
ρ (mm.)	18	18	17	18

The radii of the capillaries ranged from 1 to 2·5 mm. approx., No. 1 being the smallest. Larger capillaries are unsuitable for the test.

(*b*) Effect of rate of extrusion on ρ

Rate (cm./sec.)	0·5	0·7	1·7	2·0	2·7
ρ (mm.)	26	23	21·5	22	24

(*c*) Effect of initial length (λ_1) of column of mucus and length of column expelled before release of pressure (λ_2) on ρ

λ_1 (cm.)	13·0	9·5	9·5	9·5	6·0
λ_2 (cm.)	3·0	3·0	2·0	5·0	3·0
ρ (mm.)	12	20	20	21	28

In each of the above experiments the mucus sample used was taken from a cow at about the time of oestrus.

Flow-elasticity can be measured in a much simpler instrument than the Scott Blair viscosimeter. Experiments with secretions have shown (see Table 1) that the radius of the capillary, the pressure and the amount of extrusion before release can be varied over wide limits without effecting serious changes in the amount of recoil. The initial length of the column of secretion, on the other hand, is of some importance (see Table 1) and should be kept as constant as possible, though even differences of 100% in this are unlikely to cause confusion between oestrous and non-oestrous samples. Bearing these points in mind, a simple instrument which can be used in the cowshed for the prediction and diagnosis of oestrus has been designed and is depicted in Fig. 4.

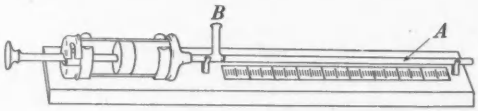

Fig. 4. The Oestroscope.

A glass capillary tube (*A*) about 11 cm. in length and of internal diameter approximately 2 mm., is cemented on to the nozzle of an ordinary 2 ml. hypodermic syringe. There is a mark 10 cm. from the open end of the tube and between this mark and the cement join, a small side-tube (*B*) is sealed in at right-angles. The tube is filled up to the 10 cm. mark by suction, keeping the side-tube closed with the finger. The instrument, which we have called the 'oestroscope', is then

clipped in a horizontal position with the 10 cm. tube covering a mm. scale and the secretion is slowly expressed (rate of about 0·5–1·0 cm./sec.) until the end of the column reaches the 7 cm. mark. The apparatus is fastened to a board of sufficient thickness to ensure that the extruded secretion is able to form a kind of blob round the open end of the tube without touching the bench. As the meniscus reaches the 7 cm. mark, the finger is removed from the end of the side-tube and the pressure immediately falls to zero. The amount of recoil, which is practically complete after a few seconds, is recorded. The experiment may be repeated without refilling, but subsequent readings will not be as accurate as the first, and if the string of extruded secretion has touched the bench or even if it is hanging from the end of the tube, recoil may be seriously inhibited. Some secretions tend to form 'salients', i.e. the meniscus is not sharply defined. This can generally be rectified by varying the rate of extrusion. Dilution of the secretions with substantial amounts of water or urine may increase the recoil (designated as ρ in mm.), but in practice the effect is seldom very marked (see Table 2). It is safer however to discard samples known to be badly contaminated.

Table 2. *Effect of diluting mucus with water on* ρ

% mucus in mixture	Sample A ρ (mm.)	Sample B ρ (mm.)	Sample C ρ (mm.)	Sample D ρ (mm.)
100	2	4	6	2
40	2	7·5	7	3
18	1	10	20	7

As with viscosity, temperature effects are slight. The whole test takes only about 1 min. to complete. The oestroscope may be washed by rinsing in warm water; drying is not important, since the secretion does not mix quickly with traces of water. For field work, the glass capillary, on which a mm. scale is etched, can be cemented into a metal shank so constructed as to fit the nozzle of any 'Record' syringe in the same way as an ordinary hypodermic needle. This makes the apparatus more portable and should considerably reduce its cost.

Recoil values determined in samples from 4 cows at regular intervals over a period covering a number of oestrous cycles are shown plotted in Fig. 5. It is clear that the flow-elasticity of the cervical mucus varies regularly during the oestrous cycle, rising to a sharply defined maximum at, or very near, oestrus. One of the cows, Clara 9, was served after ρ-values had been studied over three complete cycles; the periodicity in ρ immediately disappeared and, as in the case of the other properties studied, the values remained at the level characteristic of mid-cycle. Seventy-five days after service the cow was slaughtered and pregnancy confirmed by recovery of the foetus.

The sharply defined periodicity in the flow-elasticity of the cervical mucus, correlated with the oestrous cycle, appeared to afford a basis for an objective method of diagnosis of oestrus, particularly since this property can be measured so rapidly and easily.

In order, therefore, to investigate statistically the value of flow-elasticity measurements as a practical method for the diagnosis of bovine oestrus, numerous determinations have been made on samples from cows in various reproductive phases. These results, which are summarized in Table 4, were obtained on samples brought to the laboratory and tested within a few hours, at most, of sampling. The apparatus in its simplest form is so designed that the measurements can be done in the cowshed immediately after the sample has been taken, though if desired samples can be brought to the laboratory and left for some

hours before testing. The results in Table 3 show that the ρ-values of samples kept at room temperature overnight seldom alter appreciably.

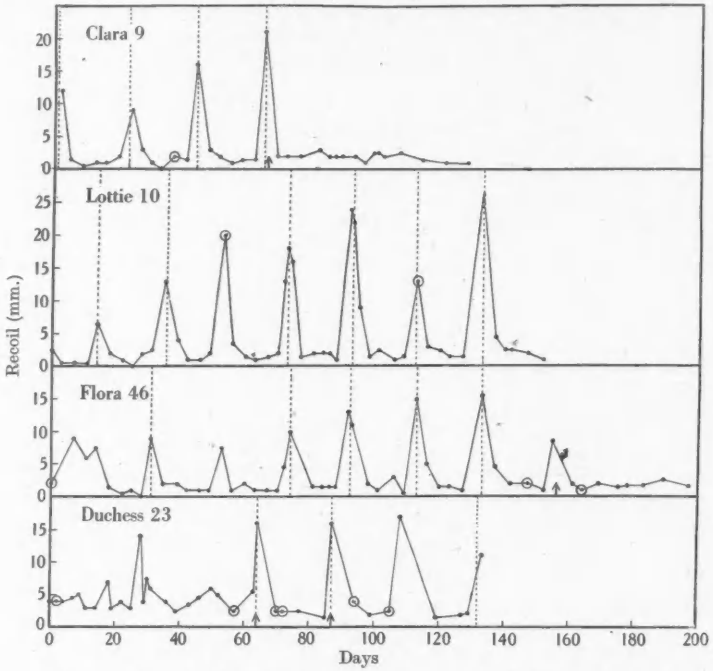

Fig. 5. Variations in flow-elasticity (ρ) of bovine cervical mucus during the oestrous cycle. Vertical broken line indicates observed oestrus and arrows denote service. Points marked with a circle are subject to a certain error due to inadequacy of sample.

Table 3. *Effect of keeping secretions for various times at room temperature on* ρ

Approx. time of keeping hr.	Sample A ρ (mm.)	Sample B ρ (mm.)	Sample C ρ (mm.)	Sample D ρ (mm.)	Sample E ρ (mm.)
1	5·5	5·0	4·0	6·0	2·0
18	5·5	—	3·5	5·5	2·0
42	5·5	4·5	4·0	7·0	—
66	—	—	4·0	15·0	—

Table 4. *Values of* ρ *in different phases of the reproductive cycle*

Group ...	I	II	III	IV	I+II
Highest ρ (mm.)	27·0	20·0	7·5	—	27·0
Lowest ρ (mm.)	5·5	6·0	0	1·0	5·5
Mean ρ (mm.)	14·7	10·9	2·4	2·5	14·0
Standard deviation	±9·6	±3·8	±1·4	±2·4	±5·8
No. of samples	41	9	143	53	50

Table 4 summarizes the results of tests on 246 samples. Group I includes samples from cows observed by the herdsman to be in oestrus within a period of 24 hr. before sampling and 60 hr. after. These arbitrary time limits are taken as

corresponding, as far as is now known, to the average duration of a high ρ. Group II consists of samples from cows in which oestrus was not actually observed by the herdsman within the prescribed time limits, but its occurrence within these limits could be inferred from previously (and in some cases also subsequently) observed oestrous periods; group III contains samples from cows which were not in, or near, oestrus as defined by the above time limits at the time of the test and which were not pregnant; group IV consists of samples from cows believed by the herdsman to be pregnant. Some, but not all, of these have been subsequently verified by recovery of the foetus.

Comparing the figures for group I and groups I and II combined, with those for groups III and IV, it is clear that ρ for samples taken at or near oestrus is significantly higher than for samples taken in mid-cycle or pregnancy. On the basis of our data, therefore, it may be concluded that a ρ-value of 5 and upwards means that when the sample was taken, the cow concerned was either actually in season or within a few hours of its onset or its end. Should the ρ-value indicate oestrus and, on the trial being made, the cow refuse to accept the bull, a second test some hours later should indicate whether or not the first test was made shortly before or soon after oestrus. The form of the curves in Fig. 5 indicates that if the first test preceded oestrus the ρ-value some hours later will have risen, and if it followed oestrus the second ρ-value will be lower.

The high standard deviation for group I is due to the facts that samples were not always taken when the cow was exactly in oestrus but might be collected at any time during the arbitrarily chosen period around oestrus, and also that the maximum ρ is doubtless not the same for all cows nor in every oestrus for any given cow.

Discussion

The criterion which we have used to define 'oestrous' samples is arbitrary and requires some explanation. The only correct criterion for a study of this kind is the test of whether or not the cow will accept the bull. For practical reasons it is not possible to apply this test nearly as frequently as would have been needed to get a statistically adequate body of data. In order to present an analysis of our data we were therefore obliged, in selecting our group of 'oestrous' samples with which to compare the 'non-oestrous' groups, to choose the arbitrary time-limits to which we have already referred.

Throughout this study, with the exception of a few cases in which the cow was served immediately after taking the sample, we were dependent on the observations of skilled herdsmen who are able to recognize oestrus from the behaviour pattern generally shown by cows in season. It must be remembered, however, that oestrus as recognized by the herdsman by no means always corresponds exactly with the bull-acceptance criterion. The allocation of borderline cases between the groups, involved an element of subjectivity; each such case was therefore considered on its merits.

Preliminary inspection of the data gave the impression that ρ begins to rise above the normal mid-cycle level (which differs somewhat for different animals) about 24 hr. before oestrus and does not return to normal until about 60 hr. after it. These figures undoubtedly differ for different animals, but after considering all the evidence they seemed to give the best limits for our statistical analysis. The fact that ρ remains high for a longer period after than before oestrus may be due to two factors: (*a*) ovulation probably takes place some time after oestrus (Brewster & Cole [1941] give approximately 13½ hr. after the end of heat and Hammond [1927] about 40 hr. after its start) and (*b*) mucus produced

at oestrus probably remains in the reproductive tract for some time after its secretion. Nothing is yet known of the effects (if any) of ovulation on the value of ρ, though it seems likely that there are such effects. It is clearly desirable to investigate this point and also to determine the way in which ρ varies hour by hour during the period of oestrus and for some time before and after. Facilities for such an exacting experiment have unfortunately not yet been available, though it is hoped that such difficulties may be overcome in the near future. In the meantime we believe that the treatment of our results on the basis just discussed does allow of the definite conclusion that ρ is highest at about the time when the cow will accept the bull and that therefore the oestroscope described provides a simple and rapid means of diagnosing oestrus.

Hitherto, there has been no adequate objective method of recognizing oestrus except putting the cow to the bull. According to Cole [1930], studies of vaginal smears afford no certain means of judging 'heat'. The presence of mucus in the smear, although associated with oestrus, is an unreliable criterion, a conclusion confirmed by Ferring [1937]. Quite frequently, especially in winter, cows show little or no external signs of oestrus even though ovulation takes place. There are several examples seen in Fig. 5 in which ρ showed a sharp maximum at a time at which, from oestrus dates and the known regularity of the oestrous cycle of the cow in question, it may be inferred that oestrus did occur despite the fact that the herdsman, although duly warned, failed to observe it. Unfortunately in all cases save two it was not possible to settle the matter definitely by serving the cows on these occasions. In one case, however, the cow stood to the bull while a semen sample was collected in an artificial vagina and in another instance the cow was served and became pregnant.

It is hoped that the oestroscope may prove particularly useful in such cases as those in which 'heat' would otherwise be missed and much valuable time wasted in getting cows into calf. Our results indicate that total N determinations would provide as reliable an indication of oestrus as the oestroscope but the latter affords a much simpler and quicker method.

Viscosity determinations, intermediate in complexity, are not so reliable, probably mainly owing to the serious effects on the viscosity of contamination with relatively small amounts of urine, such as would not cause a wrong diagnosis in the case of the other two methods.

Summary

1. Variations in total N, dry matter, viscosity and flow-elasticity of bovine cervical mucus have been studied during the oestrous cycle.

2. Total N, dry matter and viscosity reach minimum values at about the time of oestrus; flow-elasticity shows a marked maximum.

3. Variations in total N in the secretions are great enough to serve as the basis of a test for the diagnosis of oestrus. The drop in percentage of total solids at oestrus is quite insufficient to account for the differences in total N observed. It seems that soon after oestrus nitrogenous substances enter the secretion.

4. The secretions are not true fluids but a viscosity value can be determined under arbitrarily standardized conditions, and this value could serve as the basis for a test for the diagnosis of oestrus were it not for the fact that various factors, especially comparatively slight contamination of the samples with urine, seriously influence the results.

5. A simple apparatus for the determination of flow-elasticity in the field is described (the Oestroscope). An analysis of numerous determinations shows that the chances of a false diagnosis of oestrus by this means are small. The test is

easily carried out, takes no more than about 2 min. to complete and is therefore to be preferred to the other methods tried by us.

6. It is believed that the oestroscope technique should be of practical value to stock-breeders, especially for prediction of oestrus in cases where it is liable to be missed, and for its diagnosis in cases where ovulation is unaccompanied by external signs of 'heat' ('still-heat').

We should like to express our best thanks to the Agricultural Research Council for a research grant to one of us (F. H. M.), to the Earl of Iveagh and Captain G. T. Addis, and to our colleague, Mr J. Mackintosh, for generous co-operation in providing access to experimental animals. We are also grateful to Miss H. M. Scott Watson (Mrs C. C. Thiel) for taking mucus samples at the beginning of the investigation.

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121. VITAMIN METHODS

1. AN IMPROVED PROCEDURE FOR ESTIMATING VITAMIN B_1 IN FOODSTUFFS AND BIOLOGICAL MATERIALS BY THE THIOCHROME TEST INCLUDING COMPARISONS WITH BIOLOGICAL ASSAYS

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During the past two years a systematic study has been made in this laboratory of the various possible sources of error and the technical difficulties involved in the thiochrome test, based on the now well-known reaction first described by Jansen [1936]. These errors and difficulties are more serious than is generally appreciated. In an earlier paper [Wang & Harris, 1939] a procedure was described for urine, by which interference from non-specific substances and various other problems of technique were successfully overcome. Our results were controlled by biological tests, and this proof of the reliability of the method applied to urine has been further confirmed since [see accompanying paper, Harris & Wang, 1941]. The present communication describes how the technique used by Wang & Harris may be adapted for estimating vitamin B_1 in foodstuffs and in animal and plant tissues. As we have again repeatedly checked our results by biological assays, we feel considerable confidence in the method which we are now recommending.

Need for investigating potential errors in the thiochrome test

It is curious that although thiochrome determinations have been carried out by various workers, e.g. by Westenbrink & Goudsmit [1938] on animal tissues, by Wiegland [1938] and by Houston *et al.* [1940] on milk, and by Pyke [1939] on a long list of 190 food materials—little effort has been made hitherto (except for the few estimations on milk by Houston *et al.* [1940]) to test the findings by direct comparison against biological results, and even more surprising is the fact, which seems to have escaped general notice, that many of the results published are systematically low when compared with the usually accepted values as based on biological tests. This liability to error, in the absence of precautions such as those worked out in the paper, has again been forced on our notice in the results of a recent series of collaborative trials in which we were able to participate; values based on the present method agreed well with the biological tests, whereas those based on other thiochrome methods were frequently low. (For a full tabulation of these comparative results, see Wang [1941].)

That there was a need to work out a procedure at once reliable and reproducible has also been brought home to us repeatedly during the past year or two by investigators in other laboratories, who have told us of the difficulties

which they have experienced when attempting to estimate the vitamin by other methods based on the thiochrome reaction. We believe we can claim that the process described in this paper is satisfactory, since, apart from the agreement with the biological findings referred to above, it has been used for continuous routine tests for many months by three independent workers in this laboratory, and their results have tallied closely; also workers from four other institutes, who at their request were provided with details of the method during 1939 or 1940, after having experienced difficulties with other techniques, report that it has worked without trouble and given reproducible results. It has also stood the test of use by students of the advanced biochemical class at Cambridge during the past two sessions.

Comparison with azo method. The improved thiochrome test seems preferable to the alternative chemical method, based on the azo reaction [Prebluda & McCollum, 1939; Melnick & Field, 1939; cf. Kinnersley & Peters, 1934] in being more sensitive and in not requiring an elaborate process of preliminary purification in order to prepare a concentrate suitable for the reaction. We are able to determine as little as 0·03 μg. of aneurin per ml. in extracts from foodstuffs, with an error of $\pm 10\%$. With extracts containing more than 0·1 μg. of aneurin per ml., the error is usually about $\pm 5\%$.

Preliminary control tests

The thiochrome test depends on the conversion of vitamin B_1 into its oxidation product, thiochrome, under the action of alkaline ferricyanide, and on the measurement of the fluorescence produced by the latter, generally after extraction in *iso*butanol.

As the procedure for foodstuffs and tissues now to be discussed is based on the method of Wang & Harris [1939] for urine, it will be convenient first to consider the special measures taken in the latter method to overcome the various technical difficulties which arise. These have been adopted and, where necessary, modified in the present process; and in addition certain new problems have had to be faced. The three principal features of Wang & Harris's method comprised:

(1) Extraction, prior to oxidation, with *iso*butanol, to remove interfering substances.

(2) Subsequent washing of the *iso*butanol layer (containing the thiochrome) for further purification from both pigments and non-specific fluorescing substances.

(3) Visual comparison, to distinguish between specific blue fluorescence due to the thiochrome and any non-specific yellowish fluorescence.

Other precautions taken over from the method for urine include:

(4) Control of the amount of ferricyanide used, to prevent (*a*) a partial destruction of the thiochrome formed and (*b*) variable effects on non-specific fluorescent substances. The destructive effect of excess of ferricyanide is seen from Table 1.

Table 1. *Influence of variations in amount of potassium ferricyanide added*

	Test 1	Test 2	Test 3	Test 4
Vitamin B_1 present, μg.	0·40	0·40	0·40	0·40
H_2O, ml.	1·48	1·4	1·0	0·5
MeOH, ml.	2·0	2·0	2·0	2·0
NaOH, 30% solution, ml.	1·0	1·0	1·0	1·0
$K_3Fe(CN)_6$ 2% solution, ml.	0·025	0·10	0·50	1·0
Vitamin B_1 found, μg.	0·41	0·40	0·35	0·32
Recovery of vitamin B_1, %	102	100	87·5	80

(5) Omission of adsorption which generally caused a partial loss or inactivation of vitamin B_1, judged from recovery experiments on the eluate [cf. also Jowett, 1940].

(6) Treatment of the reagents used, to remove fluorescing or other interfering substances.

(7) Inclusion of a blank control, to allow for a small residuum of interference.

In work on foodstuffs two major additional problems have to be faced which do not arise with urine: (8) the preliminary digestion or extraction of the material to get the vitamin into a form suitable for the estimation and (9) the conversion of cocarboxylase into aneurin and its differentiation from the latter. These and other related questions may next be discussed.

(8, 9) *Preliminary digestion. Conversion of cocarboxylase into aneurin*

For converting cocarboxylase into aneurin, phosphatases derived from various sources can be used. Thus, Hennessy & Cerecedo [1939] used a preparation of beef kidney. This however has been criticized by Melnick & Field [1939] as giving an incomplete conversion. We have tested takadiastase [Kinnersley & Peters, 1938] and dried yeast [Melnick & Field, 1939], and prefer the former. The disadvantage of yeast is that an additional blank has to be run side by side with the test on the 'unknown' to allow for the aneurin present in the yeast itself; with takadiastase (Parke Davis) on the contrary no blank is needed as the enzyme preparation is free from vitamin B_1.

We have found that the most convenient procedure for removing any protein from the unknown, and otherwise obtaining an extract suitable for the test, is by digestion with papain. Other workers [e.g. Pyke, 1939] have used pepsin followed by takadiastase. The advantage of papain is that it can be combined with takadiastase in a single digestion at one *p*H value, whereas if pepsin is used two consecutive digestions at different *p*H values are necessary.

(10) *Partial solubility of thiochrome pyrophosphate in* iso*butanol*

Some error is caused in the estimation of the aneurin-cocarboxylase ratio in a mixture of the two, if the method adopted by most past workers is followed. This has involved a determination of aneurin both before and after hydrolysis of cocarboxylase. The increased aneurin found after hydrolysis has been taken as the cocarboxylase. This procedure is based on the assumption that thiochrome pyrophosphate, formed by the action of the K_3FeCy_6 on unchanged cocarboxylase, is insoluble in the *iso*butanol layer. But this is not strictly true. We have found that thiochrome pyrophosphate is soluble to a considerable extent in the so-called '*iso*butanol layer' which is really a mixture of *iso*butanol, methanol and water. Since thiochrome pyrophosphate shows the same blue fluorescence as thiochrome, its presence in the *iso*butanol layer causes an apparent increase in the thiochrome reading. The error thus introduced may be quite large when the amount of free aneurin is low in comparison with the cocarboxylase. This is shown in Table 2. The error is almost completely eliminated in our process by the washing of the *iso*butanol layer with water. This operation has the additional advantage that it removes excess of alkali from the *iso*butanol layer, and thereby considerably improves the stability of the thiochrome (Table 2).

Westenbrink & Jansen [1938] originally proposed to determine cocarboxylase by a process in which the preliminary hydrolysis into aneurin is omitted. The fluorescence of the *iso*butanol layer is taken as representing the free aneurin, and the fluorescence of the aqueous layer after the extraction with *iso*butanol as

Table 2. *Estimation of free aneurin in the presence of cocarboxylase. Interference from cocarboxylase removed by washing the* iso*butanol layer with* H_2O

Total amount present, as μg. of aneurin	Proportion of cocarboxylase and aneurin present		Free aneurin found, %	
	Cocarboxylase %	Aneurin %	Without washing	With washing
0·38	100	0	16	1
0·28	95	5	26	5·7
0·26	77	23	31	21
0·4	50	50	57	48

representing the cocarboxylase. The method has the objection just indicated—namely partial loss of the cocarboxylase derivative (thiochrome pyrophosphate) into the *iso*butanol; and in addition there is frequently serious interference from other fluorescent substances present in the aqueous phase. Such interfering substances are eliminated by the washing in our process.

(11) *Protective action of methanol. Optimum addition of NaOH*

Westenbrink & Goudsmit [1938], Ritsert [1938] and Karrer & Kubli [1937] all omit the use of methanol in the oxidative process. This modification has the advantage that less thiochrome pyrophosphate dissolves in the *iso*butanol layer, because of the decreased solubility of water in *iso*butanol in the absence of methanol. On the other hand it has the serious objection that in the absence of methanol accurate quantitative estimation of the vitamin is not possible owing to the destruction (presumably by oxidation) of the vitamin or of the thiochrome formed from it. Only 50–70% of the true value is found (Table 3). In our

Table 3. *Effect of MeOH on the recovery of thiochrome*

Vit. B_1 present, μg.	0·20	0·20	0·20	0·20	0·20*	0·20*	0·20*	0·20*	0·20*	0·40
H_2O, ml.	0·5	0·5	1·0	1·0	1·5	1·5	2·0	2·0	3·5	0·5
MeOH, ml.	2·0	2·0	2·0	2·0	1·5	1·5	1·0	1·0	0	2·0
NaOH, 30% solution, ml.	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
$K_3Fe(CN)_6$, 2% solution, ml.	0·04	0·2	0·04	0·2	0·04	0·2	0·04	0·2	0·08	0·08
Vit. B_1 found, μg.	0·19	0·19	0·19	0·19	0·19	0·17	0·16	0·16	0·14	0·41
Recovery, %	95	95	95	95	95	85	80	80	70	102
Vit. B_1 present, μg.	0·40	0·40	0·40	0·40	0·40*	0·40	0·40*	0·40*	0·40*	0·40*
H_2O, ml.	1·0	1·0	1·0	1·0	1·0	1·5	2·0	2·0	3·0	3·0
MeOH, ml.	2·0	2·0	2·0	2·0	2·0	1·5	1·0	0	0	0
NaOH, 30% solution, ml.	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
$K_3Fe(CN)_6$, 2% solution, ml.	0·08	0·04	0·04	0·04	0·04	0·08	0·08	0·04	0·08	0·08
Vit. B_1 found, μg.	0·41	0·40	0·40	0·40	0·38	0·40	0·37	0·20	0·24	0·20
Recovery, %	102	100	100	100	95	100	93	50	60	50

* Methyl alcohol was added afterwards to the *iso*butanol extract, to compensate for the difference in volume.

experience it is essential that methanol be added to the extent of at least 50% by volume before the addition of the NaOH, in order to ensure that there is no destruction of the vitamin (Table 3)[1]. Provided that this proportion of MeOH is

[1] In presence of MeOH higher fluorescences were obtained when the $K_3Fe(CN)_6$ was added before the NaOH, while in absence of MeOH the reverse held good. The latter procedure however gave slightly lower values and involved risk of loss of vitamin if there were delay between the additions of the reagents.

Table 4. *Effect of varying the amount of NaOH added*

	Test 1	Test 2	Test 3	Test 4
Vitamin B_1 present, μg.	0·40	0·40	0·40	0·40
H_2O, ml.	2·3	2·0	1·5	0·5
MeOH, ml.	2·0	2·0	2·0	2·0
NaOH, 30% solution, ml.	0·2	0·5	1·0	2·0
$K_3Fe(CN)_6$, 2% solution, ml.	0·06	0·06	0·06	0·06
Vitamin B_1 found, μg.	0·34	0·37	0·40	0·41
Recovery, %	85	92·5	100	102

Table 5. *Influence of time allowed for the reaction on the percentage recovery*

1 ml. aneurin solution containing 0·20 μg. aneurin treated with 2·0 ml. MeOH, 0·04 ml. of 2% $K_3Fe(CN)_6$ and 1 ml. of 30% NaOH. The mixture was allowed to stand for varying lengths of time before extraction with *iso*butanol.

Time allowed before extraction, min.	1	5	5*
Vitamin B_1 present, μg.	0·20	0·20	0·20
Vitamin B_1 found, μg.	0·19	0·19	0·19
Recovery, %	95	95	95

* 0·25 ml. of 2% $K_3Fe(CN)_6$ added in this instance.

present, the NaOH can then be safely added in the quantities necessary to effect complete conversion of the vitamin B_1 into thiochrome (Table 4). Under these conditions the thiochrome formed is found to remain undestroyed, even when the reaction mixture is allowed to stand for as long as at least 5 min. before the extraction with the *iso*butanol (Table 5).

(12) *Factors inhibiting the hydrolysis of cocarboxylase*

We have found that some animal tissues, e.g. liver, contain a factor or factors which appear to inhibit the hydrolysis of the naturally present cocarboxylase (or other precursor, if such there be). As is shown in Table 6, the hydrolysis of

Table 6. *Recovery tests after digestion for different periods of time and under varying conditions*

		Amount recovered after digestion for 1 hr.		Amount recovered after digestion for 3 hr.		Amount recovered after digestion for 24 hr.	
		μg.	%	μg.	%	μg.	%
Cocarboxylase, specimen 1	Takadiastase	80	—	—	—	82	—
Cocarboxylase, specimen 2	Takadiastase	86	—	—	—	84	—
Cocarboxylase, specimen 3	Takadiastase	70	—	—	—	72	—
Liver, specimen 1	Takadiastase	1·1	—	2·2	—	7·9	—
Liver, specimen 1 + cocarboxylase ≡ 7·1 μg. vit. B_1	Takadiastase	2·1	14	4·8	37	14·3	90
Liver, specimen 2	Takadiastase	0·44	—	—	—	—	—
Liver, specimen 2 + 10 μg. vit. B_1	Takadiastase	10·2	98	—	—	—	—
Liver, specimen 3	Takadiastase + papain	8·8	—	10·6	—	11·9	—
Liver, specimen 3 + cocarboxylase ≡ 17·5 μg. vit. B_1	Takadiastase + papain	26·7	102	29·7	109	29·7	98
Liver, specimen 3 + 17·5 μg. cocarboxylase	Takadiastase	13·9	—	26·7	—	29·7	—
Liver, specimen 4, heated for 10 min. at 100° before digestion	Takadiastase	1·8	—	—	—	8·3	—
Liver, specimen 4 + cocarboxylase ≡ 7·1 μg. vit. B_1	Takadiastase	4·2	34	—	—	15·4	100

the cocarboxylase in liver by means of takadiastase was slow and incomplete, whereas pure synthetic cocarboxylase under the same conditions was completely hydrolysed after incubation for 1 hr. The inhibition is largely removed by the incubation with papain. This is an additional reason for the method of digestion favoured by ourselves.

It does not seem that this phenomenon of 'inhibition' is due to enzymic action, e.g. to phosphorylation of the aneurin set free, for we have found that added free aneurin can be recovered quantitatively unchanged, and further that preliminary heating to destroy enzymes did not abolish the inhibition.

(13) *Masking effect of pigments on the fluorescence*

We have observed that with blood, liver and certain cereals, if the digest did not receive the preliminary extraction with *iso*butanol before oxidation, coloured substances were present which masked the fluorescence of the thiochrome in the final comparison. With some materials the interference was so

Table 7. *Removal of interfering pigments by preliminary extraction with isobutanol*

	Aneurin found, μg.		
	Without extraction	After extraction	Recovery %
Whole rat blood + cocarboxylase ≡ 7·1 μg. vit. B_1 (digested with takadiastase for 5 hr.)	0	3·8	50*
Whole rat blood + free aneurin, 7 μg. (digested with takadiastase for 5 hr.)	0	5·8	80
Maize, specimen no. 1	0	0·11	—
Maize, specimen no. 2	0	0·11	—

* The hydrolysis of cocarboxylase was probably incomplete under these conditions.

great that it completely masked the fluorescence of the thiochrome; both in the blank and the unknown only the colour of the natural pigment could be seen. Table 7 illustrates the beneficial effect of the preliminary extraction with *iso*butanol.

(14) *Differences in tints of fluorescence*

Sometimes it is observed on matching that the tint of the fluorescence in the unknown or in a control solution of pure thiochrome is distinctly different from that in the blank, e.g. the latter may be tinted white or yellow, while the unknown is more usually a clear bluish-violet. This effect arises from the destruction by the ferricyanide of certain non-specific fluorescing substances which are present along with the vitamin B_1 in the sample, but which differ from vitamin B_1 in the shade of the fluorescent tint. This oxidation by the ferricyanide does not occur in the blank, which contains no ferricyanide. The difference between tint of unknown and blank is more pronounced when the amount of ferricyanide added is in great excess. In our method the difficulty is largely overcome: first, by careful regulation of the amount of ferricyanide added so that only a slight excess of it is present; secondly, by the preliminary extraction before oxidation, with *iso*butanol, which removes the greater part of the non-specific fluorescent substances; and thirdly, by the use of suitable filters, e.g. Wratten 18 A and 49 A.

This difficulty of a difference in tint is a possible objection to all modifications of the thiochrome method. It is the chief reason why objective measurements, as with a photoelectric cell, are not necessarily better, and may in fact be far less accurate, than the direct visual comparison. With the visual comparison differences in tint may be looked for and allowed for, whereas with a mechanical recording of fluorescence they may be more easily overlooked.

Working directions

The procedure described below has been found suitable for use with a very wide range of cereal products, yeast and yeast extracts, and for meat or animal tissues generally. It has also worked well with the somewhat more limited number of fruits and vegetables so far tested (including cauliflower, spinach, potatoes, oranges.[1]

To obtain accurate results it is essential that the following process be adhered to closely. Determinations should be carried out in duplicate to check any possibility of random errors.

A. *Extraction and digestion*

The process of preliminary extraction and digestion can be varied somewhat according to the nature of the product to be examined.

(1) *For cereals.* With dry materials such as whole grain, flours, biscuits etc., grind up the specimen to as fine a state as possible and take a representative sample. With moister preparations, e.g. breads, it is often convenient to break up into small crumbs and spread out to dry in a thin layer in air at room temperature before sampling; alternatively, a representative specimen of the fresh moist crumb may be extracted and digested direct without drying. It is important not to leave the moist material standing too long before examination, as the vitamin is liable to become oxidized at an alkaline or almost neutral reaction.[2] Take 1–2 g. (or more for a relatively inert material, e.g. up to 5 g.) and heat in a conical flask with about 15 ml. of acidulated water (2 drops of conc. HCl per 50 ml. of water) at 100° on a water bath for 10 min. with continual stirring. Then add 5 ml. of 0·1 *M* acetate buffer, *p*H 4; mix and cool to 40°. Readjust the reaction accurately to *p*H 4 if necessary by addition of a drop or two of 10% HCl, using bromocresol green as external indicator. A pasty suspension results, and two alternative methods may be used for the subsequent digestion of it: (*a*) with takadiastase plus papain or (*b*) with takadiastase alone. The former is preferred as being more accurate, particularly with materials of low activity, and is also more convenient if a large number of materials has to

[1] A more detailed study of plant products is in progress.

[2] Even at the neutral or mildly alkaline reaction of commercial yeast extract, serious and progressive deterioration occurs (see Table 8).

Table 8. *Deterioration of yeast extracts on standing*

Material	Length of time kept standing	Vitamin B_1 I.U. per g.
Commercial yeast extract, 'M'	Examined on purchase	5·8
	After 3 months	3·7
	After 2 years	1·0
	After 4 years	0·8
Commercial yeast extract, 'Y'	Examined on purchase	8·2
	After 2 months	6·6

be examined simultaneously. Probably its only disadvantage compared with method (*b*) is that it takes longer to carry out, since it entails leaving the digestion mixture overnight.

(*a*) *Digestion with takadiastase and papain.* To the pasty suspension at *p*H 4 and temperature 40°, now measuring a little over 20 ml., add 0·1 g. of takadiastase plus 0·1 g. of papain. Incubate overnight at 40–45° with the addition of a few drops of toluene as preservative. Make up to 25 ml., centrifuge and discard the insoluble residue.[1]

(*b*) *Digestion with takadiastase.* To the pasty suspension at *p*H 4 and temperature 40°, add about 0·1 g. of takadiastase and incubate for some 15 min. at about 40° with occasional shaking. (After this 15 min. incubation, it may be occasionally advisable, in instances in which the original sample was not sufficiently fine and homogeneous, to centrifuge and re-extract the residue with 7–8 ml. of acidulated water at 70–80° for 15 min. with stirring, and combine the extracts and residue.) If necessary, incubate again a second time, for 1–2 hr. at 40–45°; this second incubation may be omitted for most cereals, which contain negligible amounts of cocarboxylase. Now make up the volume of extract plus residue to 25 ml. or other convenient volume. Centrifuge and discard the residue.[1]

(2) *For meat and meat products.* First remove any obvious fat from the lean, then grind 1–2 g. of the latter (more for inert materials) to a fine pulp in a mortar with one drop of conc. HCl plus a few ml. of 0·1 *M* acetate buffer (*p*H 4), using sand if necessary. Decant off the suspension into a measuring flask, washing the sand remaining in the mortar 2 or 3 times with the buffer solution. Make up the total volume of extract including residue to about 10 ml. (or other convenient volume), check the *p*H (adjusting to *p*H 4 with a drop or two of acid if necessary), and incubate with 0·1 g. of takadiastase plus 0·1 g. papain at 40–45° overnight in presence of a few drops of toluene. After cooling readjust the fluid to a convenient volume (10–25 ml.). Centrifuge the digest, and discard the insoluble residue.[2]

(3) *For yeast and related products.* Take 1 g. or more of the finely ground sample, add about 15 ml. of acidified water and adjust the reaction to *p*H 4 with buffer. Digest with papain and takadiastase as described under paragraph 1 (*a*) but with the addition of 1–2 ml. of toluene to inactivate the yeast; make up to a suitable volume (e.g. 50 ml. for specimens of dried brewer's yeast of average potency, *ca.* 20 I.U. per g., or in proportion) and centrifuge.

In the case of *yeast extracts*, first dilute the extract until the concentration of vitamin B_1 is approximately 0·5–1·0 μg. per ml. Adjust the reaction to *p*H 4 with acetate buffer or HCl and incubate overnight, as in paragraph 1 (*a*) above, with takadiastase and papain in presence of a few drops of toluene; make up to volume and centrifuge.

(4) *For plant tissues*, e.g. potatoes, spinach, cauliflower etc. Grind about 10 g. of the sample with 2–4 drops of conc. HCl to a fine pulp in a mortar. Transfer the suspension to a conical flask, make up the volume with water to about 30–40 ml. and immerse in boiling water for 10 min. Cool, adjust reaction to *p*H 4, then digest with takadiastase and papain, make up to volume and centrifuge, as in paragraph 1 (*a*).

[1] *Calculations of volume.* In determining the total volume of the digest it is best to include the small undigested residue in the measuring flask. This is more accurate than attempting to allow for it, since imbibition of water by the protein residue may greatly increase its apparent bulk, so that any supposed correction for the insoluble residue would in fact be fictitiously high.

[2] See above footnote.

(5) *For milk, milk powder, egg products.* With milk powder, first dilute by mixing about 5 g. with about 30 ml. of water; milk can be used without preliminary dilution, using about 20–30 ml. Adjust the reaction to *p*H 4, and proceed as in paragraph 1 (*a*).

B. *Preliminary washing with* iso*butanol*

With a pipette, transfer 5 ml. of the clear digest to a centrifuge tube. Extract by shaking for 1 min. with an equal volume of *iso*butanol[1] which has been previously saturated with water. Centrifuge, and draw off the upper *iso*butanol layer, using a pipette or glass tube provided with a teat. Discard the *iso*butanol layer. Note the final volume of the aqueous layer.

C. *Oxidation to thiochrome*

Transfer by means of a pipette 1 or 1·5 ml. portions of the aqueous layer into two graduated glass-stoppered cylinders of 25 ml. capacity. If 1 ml. sample is used, add water to make it up to 1·5 ml.: this has the advantage of facilitating the subsequent separation of the layers (1·5 ml. samples can be used with less active products). Add to each cylinder 2 ml. of methanol. Add one drop or more, depending on the amount of reducing substances present, of a 2% solution of potassium ferricyanide to one cylinder only (the 'unknown'). The second cylinder, without the $K_3Fe(CN)_6$, is kept as the 'blank' and, apart from the omission of this reagent, is to be treated in precisely the same manner as the 'unknown'. (It may be worth while, though not absolutely necessary, to run a preliminary trial as described by Wang & Harris [1939] to determine the optimum amount of $K_3Fe(CN)_6$ to add to the 'unknown'.) In a third cylinder (the 'control') place 1 ml. of the diluted standard (see below), make up to 1·5 ml. with water, and add ~~three drops~~ 2 ml. of methanol and one drop only of the ferricyanide solution. Then add 1 ml. of a 30% solution of NaOH to each of the three cylinders. Mix, allow to stand for 1 min. and then add 10 ml. of *iso*butanol[1] to all three to extract the thiochrome produced. Shake the cylinders vigorously for 2 min. and then allow the layers to separate.

D. *Washing with water*

Remove the bottom aqueous layers by means of a long narrow tube provided with a rubber teat. Add 3 ml. of water to each cylinder, now containing the *iso*butanol layers only, and shake for another minute. Allow the layers to separate, then with the tube transfer the three *iso*butanol layers to dry conical flasks and add 2 ml. of 96% ethanol to each, to clarify.

E. *Comparison of fluorescence*

Measure 10 ml. of the clear extract from the 'unknown' into one test tube and 10 ml. from the 'blank' into another. Care must be taken that the test tubes used are made from glass which does not fluoresce in the rays of the mercury-vapour lamp. Match the fluorescence visually without delay. For this purpose place the two test tubes side by side in front of the Wood's glass window of the ultra-violet lamp inclining them towards the body at an angle of approximately 60° to the horizontal so that the eye can look into the tubes. Add the standard 'control' solution little by little from a graduated pipette to the 'blank' until the fluorescence exactly matches that in the 'unknown'. To check possible uneven illumination of the two tubes, their positions should be reversed frequently

[1] The *iso*butanol is redistilled from the pure reagent. All reagents used, including filter papers, must be tested for freedom from fluorescence [see Wang & Harris, 1939].

during the matching. Add to the 'unknown' an amount of *iso*butanol equal in volume to the amount of the standard added to the blank, so as to make the final volumes in the two tubes the same. The contents of the tubes may be mixed after each addition by closing the tube with the thumb and inverting it twice. It is important that the matching should relate entirely to the bluish fluorescence of the thiochrome in the 'unknown', without respect to any superadded yellow or other colour present in the blank. To obviate this difficulty it is advisable when the tints of fluorescence are different, to use, in addition to the Wood's glass, a Wratten 18 A filter. This filter cuts off the visible light more completely and allows the passage of light waves of 300–400 mμ, which corresponds with the absorption maxima of thiochrome, 368 and 369 mμ. Alternatively, a Wratten 49 A filter may be used in place of the 18 A, when the latter is found inadequate. This filter cuts off any yellow or green colour and allows a better matching. It should, however, be held between the eye and the test tubes and not between the lamp and the test tubes as with the 18 A filter. It is preferable to use filter 18 A rather than 49 A for most purposes.

A little experience is needed to become proficient in making precise matches of fluorescences. The following hints may be found helpful. On entering the dark room it is advisable to allow a few minutes to elapse before attempting a reading, in order to enable the eyes to become adapted.[1] The tubes, which, as already mentioned, are inclined at an angle of 60° to the horizontal, are held side by side with their bottoms resting on a platform covered with mat black cloth to form a suitable dark background for viewing the fluorescence. The observer should look down the tubes and make a *quick decision* which of the two tubes shows the greater fluorescence. It is inadvisable to look for longer than 3 sec. at a time. Reverse the position of the tubes and confirm the finding. Repeat this procedure after each addition of the control solution until a match is obtained. Finally, add a small excess of control and confirm that the fluorescence is then slightly greater.

F. *Differentiation of cocarboxylase and aneurin*

The above method gives the total vitamin B_1 both as free aneurin and cocarboxylase. Most cereals contain little or no cocarboxylase. Meat products and yeasts contain both. To estimate them differentially proceed as follows. First prepare an extract as directed in paragraph A above (amount as specified), omitting the digestion. Transfer the extract plus residue at about *p*H 4, to a conical flask. Immerse in boiling water for 5 min. (to inactivate phosphatases) and cool to room temperature. Add a drop of acid or alkali as necessary to bring the reaction to *p*H 4, adjust to a convenient volume (e.g. 10–25 ml.). Determine the free aneurin on a portion of the centrifuged extract as described above (B to E), but without the preliminary digestion. Digest the remaining portion with takadiastase and papain as before, and estimate the aneurin content again. The difference between the values before and after digestion gives the amount of cocarboxylase.

G. *Preparation of aneurin standard*

The standard solution of vitamin B_1 can be conveniently prepared from 'Benerva' ampoules by diluting a measured amount with water and acidifying with a drop of conc. HCl. Prepare, by such means, a stock solution containing

[1] The mercury vapour lamp should be completely enclosed in a light-proof case, save only for the small horizontal window of Wood's glass between it and the experimental tubes; otherwise the room should be completely dark.

20 μg. of aneurin per ml. Dilute a portion of this to a concentration of 4 μg. per ml. and use 1 ml. of the latter for the 'control' (paragraph C). Keep the stock solution in a refrigerator. The strength of the stock solution should be checked occasionally by comparing with a preparation of aneurin of known purity, preferably a specimen of the international standard.[1] The stock solution can be kept in the refrigerator for a few days, after which it should be replaced by a freshly prepared supply.[2]

H. *Calculation*

Let g = wt. of sample taken, g.
V = total vol. of extract after digestion, ml.
r = ratio of the vol. of extract after preliminary washing with *iso*butanol, to vol. before washing.
v = vol. of final extract used for oxidation.
x = vol. of thiochrome standard required, ml.
y = amount of aneurin taken for oxidation in diluted standard, μg.

Then aneurin content of the sample $= \frac{xy}{10} \times \frac{V}{v} \times \frac{r}{g}$ μg. per g.

Example. A specimen weighing 2 g. was taken for analysis. The total volume of extract after digestion was 25 ml.; the ratio of volumes after and before washing with *iso*butanol was 5·5/5; the volume of the extract used for oxidation was 1 ml.; the volume of thiochrome standard used in the matching was 1·5 ml., and the amount of aneurin standard taken for oxidation was 4 μg. Thus, the amount of aneurin in the sample $= \frac{1{\cdot}5 \times 4}{10} \times \frac{25}{1} \times \frac{5{\cdot}5}{5} \times \frac{1}{2} = 8{\cdot}3$ μg. per g.

Routine method

In routine work when large numbers of samples have to be analysed together, much effort can be saved by having a battery of cylindrical separating funnels which can be fitted on to a special rack and shaken simultaneously, so that operations C and D can be done very expeditiously.[3]

Under ordinary working conditions about five separate digests can be conveniently examined in 1½ hr. by one worker, or 20 in a day, while with the 'battery' equipment just mentioned, the number can be still further increased and the labour reduced.

Modified procedure for some special products

The procedure described above should suffice for all the more important carriers of vitamin B_1, including cereals, yeast and yeast derivatives, meat products etc. A fuller study of fruits and vegetables is still in progress, but no difficulty has been experienced with any of those so far examined, and the thiochrome values found for them agree well with those determined biologically (see below). When working with new types of products, probably the only two problems likely to arise are those relating to the preliminary digestion and to

[1] It is worth noting that the international standard preparation, at first described as the chloride-hydrochloride-monohydrate, is in reality nearly anhydrous. By definition, the activity of this preparation is 333,333 I.U. per g. Hence the activity of the chemically pure chloride-hydrochloride-monohydrate, as available commercially and corresponding with the specification of the 'Third Addendum to the British Pharmacopoeia, 1932 (1940)' is 320,000 I.U. per g., i.e. a difference of about 4%, or almost within the experimental error of the method at its best.

[2] Comparisons with preformed thiochrome are not recommended, it being obviously more accurate to treat the vitamin B_1 standard of reference in the same way as the unknown.

[3] Stopcocks should be sealed by moistening with a drop of water (*not* grease).

interference from pigments or non-specific fluorescing substances. In case of any doubt it is useful to add a known amount of the vitamin to the unknown and test the proportion recovered. Only rarely, when there is obstinate interference from pigments or non-specific fluorescence, should it be necessary to have recourse to preliminary adsorption of the vitamin to free it from these other substances. This seems to be best avoided when possible because of the danger of incomplete adsorption or elution. But, in actual practice, almost the only difficulty in matching which we have encountered when using the above methods has been with deeply pigmented autoclaved products, e.g. alkali-autoclaved marmite. Inadequate extraction or digestion is perhaps a more serious source of error. It should be specially guarded against with desiccated products which seem liable to prove exceptionally resistant to liberation of the vitamin (see further below).

Biological comparisons

Probably the most searching test of the accuracy of a chemical method is to check the results against those obtained by biological assays. We have carried out systematic comparisons of this kind on most classes of foodstuffs, including yeast and yeast extracts, cereals (whole grains, flours, breads, biscuits, etc.), fruits and vegetables, dairy products, fish and meat.

The majority of the biological tests were done by the bradycardia method [Drury & Harris, 1930; Birch & Harris, 1934; Leong & Harris, 1937; Baker & Wright, 1938], the advantages of which have been discussed by several recent investigators [e.g. Todd & Bergel, 1937; van Veen, 1937; Karrer & Kubli, 1937; Pedersen, 1938; Lunde, 1938; Lunde *et al.* 1938; 1939; Leong, 1939, 1, 2; Pannekoek-Westenburg & van Veen, 1939; Yang & Platt, 1939]. Frequently, as a further check, other biological methods were used in addition, viz. cure of convulsions in rats, or growth rates of rats [methods as in Birch & Harris, 1934; Harris, 1941].

In all the biological tests the procedure was to feed at least two and generally three levels of the 'unknown' and three of the standard (crystalline aneurin chloride hydrochloride), with generally about six animals at each level. A dose-response curve for the standard was constructed for each separate experiment, and the amounts of vitamin B_1 present in the various doses of the 'unknown' were read off the curve from the corresponding average responses. The accuracy of the bradycardia method under such conditions is such that the standard error of the mean is about ± 5–10% [Leong & Harris, 1937; Lunde *et al.* 1938; 1939; Pannekoek-Westenburg & van Veen, 1939; Harris, 1941]. A similar order of accuracy attaches to the two other methods used [Harris, 1941].

Summaries of the experimental data are to be found in Table 9. Values entered on the same line indicate that the comparison has been made on the identical specimen. It will be seen that, for the sake of further cross-reference, a few additional values are given, either where different biological methods have been compared on the same specimen without direct chemical check or where the chemical or biological results are useful for indicating the range of variations to be expected for a particular product or class of foodstuffs.

The products examined cover the widest possible range of activities for known foodstuffs, from the very high figure of 190–200 I.U. per g. for a dried yeast of special potency down to such negligible values as 0·2 I.U. per g. or less for such products as white bread, herring, egg white. The parallelism between the chemical and biological values seems highly satisfactory, and no serious discrepancy is apparent for any single class of the foodstuffs so far examined.

Table 9. *Comparison of chemical and biological results. Entries arranged by groups, roughly in descending order of activities*

Ref. no.*	Material		Vitamin B_1, I.U. per g. Thiochrome method	Bradycardia method	Growth method 14 days	Growth method 21 days	Cure of convulsions
	YEASTS AND YEAST PRODUCTS:						
1	Yeast, high potency, dried		200	191	—	—	—
2	„ high potency (74% H_2O)		57	66	—	—	—
3	„ high potency		67	—	—	—	—
4	„ *torula utilis*		7·5–9·0	—	—	—	—
5	„ brewers' (Guinness)		12	—	—	—	—
6	„ brewers' (dried, for test)		—	18·8	—	20	19·3
7	Yeast extract, 'M', stored		4·8	5·6	—	—	—
8	„ 'M', retail		4·6	7–8	7	6·3	5·1
9	„ 'M', retail		7·0	8·9	—	—	—
11	„ 'M', special		—	8·3	—	9·1	8·7
12	„ 'Y', retail		8·0	—	9·7	9·1	6·6
13	„ 'Y', wholesale		13·4	13·4	—	—	—
	PORK AND BACON:						
14	Pork, raw, *dried powder*		7·67	9·0	—	—	—
15	„ raw, *dried powder*		8·07	7·6	—	—	—
16	„ roast, *dried powder*		5·13	4·7	—	—	—
17	Bacon		4·0	3·7	—	—	—
	CEREALS:						
	WHEAT AND WHEAT PRODUCTS:						
18	Wheat germ, proprietary		9·5	8·4–9·6	—	—	—
19	„ special		—	7·0	—	7·2	6·9
20	„ retail		—	6·0	—	—	—
21	Middlings		—	4·5	—	—	—
22	Bran		—	3·6	—	—	—
23	Whole meals (different varieties and countries	specimen 1	1·7	1·73	—	—	—
24	„ of origin)	specimen 2	1·3	1·27	—	—	—
25	„	specimen 3	1·3	1·20	—	—	—
26	„	specimen 4	1·1	1·48	—	—	—
27	„	specimen 5	1·3	1·35	—	—	—
28	„	specimen 6	1·1	1·23	—	—	—
29	„	specimen 7	1·0	1·0–1·1	—	—	—
30	„	specimen 8	1·2	1·2–1·4	—	—	—
31	„	specimen 9	1·0	1·0–1·3	—	—	—
32	Whole meals (same variety, different soil	specimen *a*	0·97	—	—	—	—
33	„ conditions)	specimen *b*	1·3	—	—	—	—
34	„	specimen *c*	0·87	—	—	—	—
35	„	specimen *d*	1·2	—	—	—	—
36	„	specimen *e*	1·0	—	—	—	—
37	Flour, wholemeal, commercial 1		1·2	—	—	—	—
38	„ wholemeal, commercial 2		—	1·5	—	—	—
39	„ 'Hovis'		—	1·2, 1·3	—	—	—
40	„ white		—	0·2	—	—	—
41	„ straight run, bleached		0·29	—	—	—	—
42	„ straight run, unbleached		0·26	—	—	—	—
43	Bread, white, retail, various		0·22–0·24	—	—	—	—
44	„ white, fortified, formula 1		0·44	0·48	—	—	—
45	„ white, fortified, formula 2		0·4	0·47	—	—	—
45*a*	„ white, fortified, dried crumb, formula 1		0·74	0·77	—	—	—
45*b*	„ white, fortified, dried crumb, formula 2		0·60	0·61	—	—	—
46	„ yeast-enriched (Proprietary)		0·7	—	—	—	—
47	„ yeast-enriched (1·65 g./100 g.)		1·42	1·45	—	—	—
48	„ yeast-enriched (1·20 g./100 g.)		1·49	1·47	—	—	—
49	„ yeast-enriched (0·53 g./100 g.)		0·56	0·61	—	—	—
50	„ wheatmeal, dried crumb		1·08	1·1	—	—	—
51	„ wheatmeal, dried crumb, special formula		0·98	1·1	—	—	—
52	„ whole wheat, *dried powder*		0·80, 0·87	1·31	—	—	—
53	Wheatmeal biscuits, raw materials (1)		0·67	0·78	—	—	—
54	„ raw materials (2)		0·80	0·74	—	—	—
55	„ baked with $(NH_4)_2CO_3$, experimental (1)		0·67	0·71	—	—	—
56	„ baked with $(NH_4)_2CO_3$, experimental (2)		0·60	0·56	—	—	—
57	„ baked with $(NH_4)_2CO_3$, commercial (1)		0·70, 0·75	0·77	—	—	—
58	„ baked with $(NH_4)_2CO_3$, commercial (2)		0·60, 0·62	0·92	—	—	—
59	„ baked with $(NH_4)_2CO_3$, commercial (3)		0·99	1·0	—	—	—
60	„ baked with $NaHCO_3$ and $(NH_4)_2CO_3$ experimental (1)		0·25	0·17	—	—	—

Table 9 (*cont.*)

Ref. no.*	Material	Vitamin B_1, I.U. per g. Thiochrome method	Bradycardia method	Growth method 14 days	Growth method 21 days	Cure of convulsions
	WHEAT AND WHEAT PRODUCTS (*cont.*):					
61	Wheatmeal biscuits, baked with $NaHCO_3$ and $(NH_4)_2CO_3$ experimental (2)	0·30	0·29	—	—	—
62	" baked with $NaHCO_3$ commercial	0·3	≷0·3	0·34	0·37	—
62*a*	" baked with yeast	1·4	—	—	—	—
	OATS:					
63	Whole grain	1·8	—	—	—	—
63*a*	Prepared oats, various specimens	1·7–1·8	—	—	—	—
	BARLEY:					
64	Meal, varying soil conditions	0·43–0·67	—	—	—	—
65	Pearl, retail (1)	0·44	0·62	—	—	—
66	" retail (2)	0·44	0·41	—	—	—
67	" retail, various specimens	0·41–0·48	—	—	—	—
	FRUITS AND VEGETABLES:					
68	Spinach, dried powder	1·53	1·69	—	—	—
69	Orange juice, partly evaporated	0·66	0·64	—	—	—
70	" dried	5·4	—	—	—	—
72	" fresh	0·17	—	—	—	—
73	Potato, dried powder	0·57	1·03	—	—	—
74	" Majestic	0·3	0·50	—	—	—
75	" Majestic	0·32	0·39	—	—	—
77	" King Edward	0·28	—	—	—	—
79	Grass	0·52	—	—	—	—
80	Brussels sprouts	0·28	—	—	—	—
81	Cauliflower	0·26	0·34	—	—	—
	DAIRY PRODUCTS:					
82	Eggs (hen), yolk, raw, dried	2·35	2·73	—	—	—
83	" yolk, raw, fresh	0·50	—	—	—	—
84	" yolk, raw, fresh	0·50	—	—	—	—
85	" yolk, raw, fresh	0·54	—	—	—	—
86	" whole, raw	0·30	—	—	—	—
87	" whole, vacuum ice-dried, reconst.	0·30	—	—	—	—
88	" white, cooked	0·05	<0·1	—	—	—
89	Milk (cow's) powder, skimmed	0·85	0·89	—	—	—
90	" fresh	0·15	—	—	—	—
91	" fresh	0·12	—	—	—	—
92	" fresh	0·11	—	—	—	—
93	" fresh	0·10	—	—	—	—
94	" colostrum	0·15	—	—	—	—
95	" colostrum	0·09	—	—	—	—
	LIVER:					
96	Beef, roasted, dried powder	0·7	1·27	—	—	—
97	" raw, dried powder	0·93–1·1	1·67–1·74	—	—	—
98	Sheep, raw, fresh (4 samples)	0·65–1·4	—	—	—	—
99	Calf, raw, fresh (6 samples)	0·38–0·75	—	—	—	—
	See also: Pork and bacon.					
	FISH PRODUCTS:					
100	Fish 'drip', concentrated	0·30	0·41	—	—	—
101	Herring, muscle, fresh, raw	0·03	0·04	—	—	—
	CANNED FOODS:					
102	Bacon, canned	—	1·13	—	—	—
103	Ham, sliced, after storage	—	0·48	—	—	—
104	Meat and vegetable rations	—	0·22	—	—	—
105	" "	—	0·15	—	—	—
106	" "	—	<0·10	—	—	—
107	" "	—	<0·10	—	—	—
108	Meat, preserved	—	0	—	—	—
109	Potatoes, canned	—	0·12	—	—	—
110	Beetroot, canned	—	0·12	—	—	—
111	Carrots, canned	—	0·17	—	—	—

* Reference numbers in column 1 relate to corresponding entries in Fig. 1.

Table 10. *Agreement between chemical and biological values. Statistical analysis*

				No. of cases	% of total no.
Total no. of specimens compared				55	100
Chemical and biological results differing by			0–10%	32	58
,,	,,	,,	10–20 ,,	10	18
,,	,,	,,	20–30 ,,	6 (*a*)	11
,,	,,	,,	>30 ,,	7 (*b*)	13
,,	,,	,,	0–10 ,,	32	58
,,	,,	,,	0–15 ,,	41	75
,,	,,	,,	0–30 ,,	48 (*a*)	87
,,	,,	,,	>30 ,,	7 (*b*)	13

(*a*) Of the 6 cases where there was a difference of 20–30% between the chemical and biological values, all but 2 were low in vitamin B_1 (<1 I.U. per g.).

(*b*) Of the 7 cases with a difference greater than 30%, all were low in vitamin B_1 (<1 I.U. per g.), or were desiccated products for which the method of extraction employed was probably inadequate.

Excluding desiccated products and foods with negligible vitamin B_1 ($\leqslant$0·3 I.U. per g.) the statistical analysis is as follows:

				No. of cases	% of total no.
Total no. of specimens compared				39	100
Chemical and biological results differing by			0–10%	27	69
,,	,,	,,	10–20 ,,	8	21
,,	,,	,,	20–30 ,,	3	7·7
,,	,,	,,	>30 ,,	1	2·5
,,	,,	,,	0–10 ,,	27	69
,,	,,	,,	0–15 ,,	34	87
,,	,,	,,	0–30 ,,	38	97·5
,,	,,	,,	>30 ,,	1	2·5

In 75% of cases the chemical and biological tests agreed within an error of 15%, or in 87% of cases within 30%. Fuller statistical data are given in Table 10.

It seems significant that in virtually all instances where the difference between the chemical and biological results was at all serious (>15%) the food was either relatively poor in the vitamin—so that an appreciable error was only to be expected—or else was a desiccated product for which the usual method of extraction was inadequate. Again, some experimental fluctuation is inevitable in all biological methods; and, in fact, in several instances a repetition of the tests, using if necessary a larger number of animals, has given more complete agreement. We have to recognize, however, that where there has been any divergence between the chemical and biological results it has almost invariably been in the direction of the former being lower, and the latter higher.[1] The principal reason for this, we believe, is that the known sources of error in the chemical method—particularly incomplete extraction and digestion—tend to cause low figures if not adequately controlled. On several occasions we have traced unsatisfactory recoveries to the use of faulty technique for the extraction or digestion. Another partial explanation is that in the biological method, fat and protein or other ingredient may have a vitamin B_1-sparing action, so that the antineuritic potency of a food is not necessarily strictly identical with its vitamin B_1 content. Further work is needed on this latter point. It may for example prove to be advisable, when testing biologically foodstuffs poor in the vitamin but rich in fat or protein, to prepare an extract for dosing. However, these minor irregularities do not affect the validity of our main conclusion.

[1] The difference between the chemical and biological values was, as an average, −6%, or as a median −4 to −5% (excluding desiccated products and foods containing negligible vitamin B_1, $\leqslant$0·3 I.U. per g.); or average −9%, and median −8 (if all determinations are included).

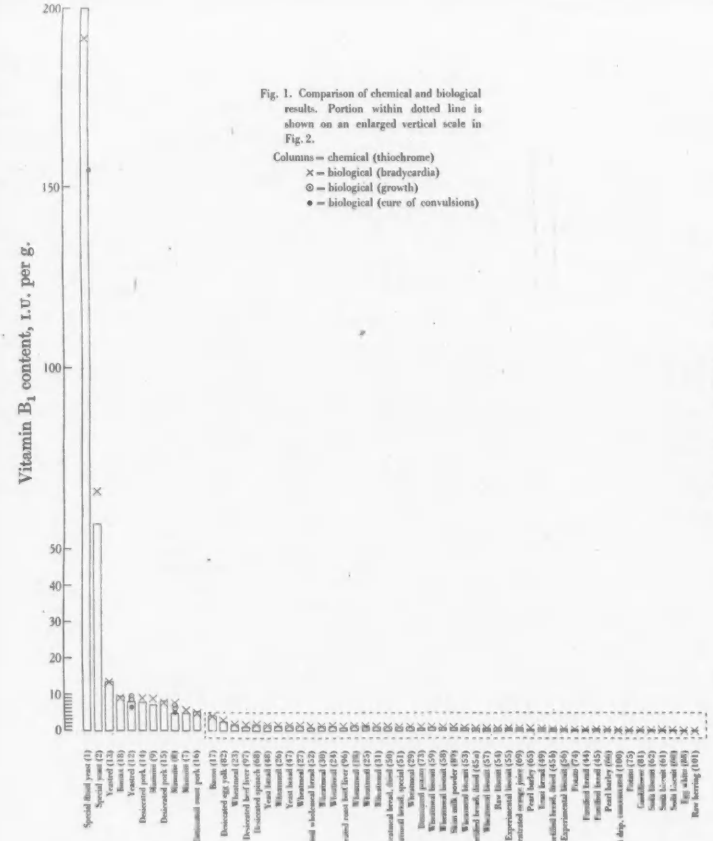

Fig. 1. Comparison of chemical and biological results. Portion within dotted line is shown on an enlarged vertical scale in Fig. 2.

Columns = chemical (thiochrome)
× = biological (bradycardia)
◎ = biological (growth)
• = biological (cure of convulsions)

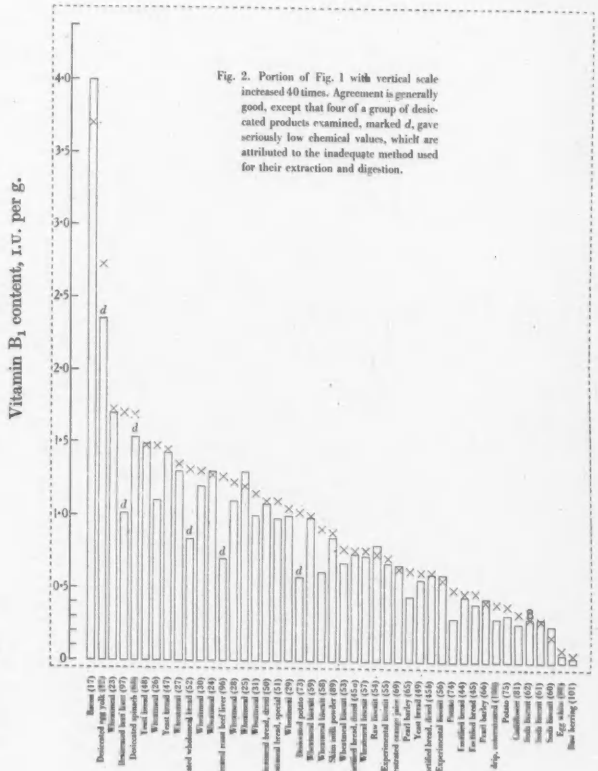

Fig. 2. Portion of Fig. 1 with vertical scale increased 40 times. Agreement is generally good, except that four of a group of desiccated products examined, marked *d*, gave seriously low chemical values, which are attributed to the inadequate method used for their extraction and digestion.

The degree of parallelism between chemical and biological findings is perhaps best shown graphically (Figs. 1 and 2). The conclusion seems justified that the improved chemical method, obviating as it does the various sources of error mentioned in the early part of the paper, does give a reliable measure of the vitamin B_1 present in foodstuffs and other animal or vegetable tissues.

Summary

1. A comprehensive study has been made of the numerous sources of error encountered when the thiochrome reaction is applied to the quantitative estimation of vitamin B_1 in foodstuffs and animal and vegetable tissues.

2. An accurate procedure, based on the method of Wang & Harris [1939] for urine, has been worked out. Special features include (*a*) a preliminary process of extraction (varying according to the nature of the product), (*b*) digestion with papain and takadiastase (for conversion of cocarboxylase into aneurin, breaking down proteins, polysaccharides, etc. and removing inhibitors), (*c*) washing of the digest with *iso*butanol (to remove interfering substances), (*d*) omission of adsorption (which in our experience caused variable losses), (*e*) conversion into thiochrome in presence of methanol, and with addition of the $K_4Fe(CN)_6$ before the NaOH (in order to protect against loss by oxidation), (*f*) washing of the thiochrome layer with water (to permit accurate differentiation between cocarboxylase and free vitamin B_1, and to eliminate non-specific fluorescent substances and pigments), (*g*) visual comparison of fluorescence with the aid of light-filters and blank controls (to detect differences of tint).

3. To test the reliability of the method, systematic comparisons with biological tests have been carried out on over 50 foodstuffs, including wheatmeals and flours, other cereals, breads and biscuits, yeast preparations, meat and animal tissues, dairy products, and a few vegetables, fruits and fish. The agreement has been good, the products examined covering the wide range of activities of 200–0·03 I.U. per g. In 75% of instances the biological and chemical values differed by less than 15%, or in 87% of instances by less than 30%; the larger percentage errors were found only with foods poor in the vitamin, or with a group of desiccated products which were difficult to extract. Where there were detectable differences the trend was for the chemical values to be lower than the biological rather than *vice versa* (average difference = −6%).

4. With 0·1 μg. of aneurin per ml. chemical results can be duplicated with an error of about ±5% and with 0·03 μg. per ml. with an error of ±10%. The procedure is easy to work, and upwards of 20 specimens can be examined in a working day.

5. Erroneously low values given by certain other modifications of the thiochrome test are explained.

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122. VITAMIN METHODS

2. A NOTE ON VITAMIN B_1 IN URINE AS DETERMINED CHEMICALLY AND BIOLOGICALLY

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Wang & Harris [1939] described a method for estimating vitamin B_1 in urine by a modification of the thiochrome reaction and found that their results agreed well with those determined biologically. Hills [1939] claims to have improved the procedure, yet states that the results by his method are 30% lower on the average than those determined by him according to our method. This apparent discrepancy has prompted us to carry out yet another series of biological comparisons on urine. The new determinations increase our confidence in the reliability of our method.

Two urines from normal subjects have been tested, one having a relatively high and the other a low content of vitamin B_1. For the biological determination it was necessary to adsorb the vitamin before dosing. For this purpose the urines were diluted 3–4 times with water, adjusted to *p*H 4 and treated with 'clarit' clay, 10 g. per 1600 ml. of diluted urine. The urines were analysed by the thiochrome method before and after adsorption, the percentage of the vitamin adsorbed being found by difference. In one instance 86% was shown to be adsorbed and in the other 82%. In the biological tests the adsorbates were administered to the rats directly. As the biological and chemical values both refer to the adsorbate, the small amount lost on adsorption does not enter into the comparison. Normally it would be included in the chemical test, which we carry out on the whole urine without prior adsorption [cf. however, Hills, 1939; Jowett, 1940].

The bradycardia method was used for the biological tests and the results are summarized in Table 1. To make the check as accurate as possible a fairly large number of duplicate doses were tested at each level. To equalize conditions for the rat, the solution of vitamin B_1 used as the standard was not administered as such but was first poured on to specimens of 'clarit' clay identical with those used for the urine adsorbate. The dose-response curve used for calculating the results is given in Fig. 1.

The technique for the thiochrome test was the same as that previously described.

Occasionally, especially with stale specimens, the final matching of fluorescence is found to be difficult. Sometimes, in such instances, a preliminary treatment with permanganate may help to improve the end point by removing the blank fluorescence without significant destruction of the vitamin B_1. This procedure can only be used with safety, however, with urines which are not too strongly reducing, otherwise appreciable destruction of the vitamin does occur. The working directions are as follows: before washing with water-saturated *iso*butanol, add 2 drops of conc. H_2SO_4 to 5 ml. of the urine. Then add a 1% solution of $KMnO_4$ drop by drop with continual stirring, allowing complete decoloration before the next drop is added. Discontinue the addition when a faint pink colour appears which persists for about 1 min. Add one drop of 0·5% H_2O_2 solution and continue as in the usual procedure.

Table 1. *Biological tests*

Sample	Vit. B_1 content of urine, I.U./ml. by thiochrome test	No. of I.U. administered according to thiochrome test	Days cured: Individual animals	Days cured: Av.	Activity as read from reference curve: Per dose given	Activity as read from reference curve: Per 1 I.U. given	Biological value found as % of chemical value
Urine 6, M. L. W.	0·154	1	2·25, 0, 1·5, 1·25, 1·25, 2	1·38	1·25	1·25	
		2	1·25, 1·75, 2·25, 3·25, 2·25, 2	2·17	2·09	1·05	
		3	5·75, 2·5, 3, 1·25, 2·5	3·00	3·00	1·00	
					Av.	1·10	110
Urine 7, W. Y. L.	0·40	1	2·25, 0, 1·5, 1·25, 1·25, 1·5	1·29	1·15	1·15	
		2	2·5, 2·25, 2·25, 1·5, 2, 1·5, 2	2·00	1·90	0·95	
		3	3·75, 2·5, 3, 2·75, 2·5, 2·25	2·79	2·75	0·92	
					Av.	1·01	101
International standard	—	1	0, 1·25, 0, 2, 1, 1·25, 2, 1·25	1·09			
		2	2·25, 3, 1·25, 2, 2, 4, 3·75, 1·25, 1·5	2·33			
		3	2·75, 3, 2·25, 3, 3, 1·25, 2·5, 3·75, 4·5, 3·25, 1·5	2·82			

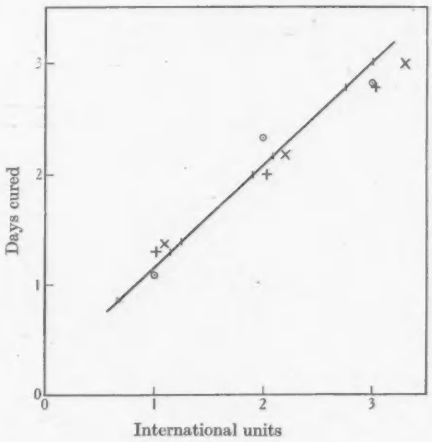

Fig. 1. Dose-response curve. ⊙ = Standard. × = Urine No. 6. + = Urine No. 7.

Agreement between the chemical and biological findings (Table 1) seems very good. For one urine the biological value is 110 % of the chemical and for the other it is 101 %. In the earlier tests [Wang & Harris, 1939], on 5 specimens of urine, the biological values were 100, 121, 100, 89 and 128 % of the chemical values, making an average of 108 %, or an average of 107 % for all 7 specimens in the two investigations.

That slightly lower results are given by the thiochrome test than by the biological test accords with our general experience of other animal and plant materials and of foodstuffs [Harris & Wang, 1941]. Methods like those of Hills which give still lower chemical results may therefore be expected to be all the more in error—viz. to the extent of more than 30%, to judge from the differences between the results given by the two methods as published by Hills.

Jowett, who criticized the uncertainties in the method of Hills, diminished the error by substituting synthetic zeolite for activated earth. But even with this improvement, various possible sources of error remain—including incomplete adsorption and elution—and as Jowett himself concedes, his procedure, although convenient and simple, will not estimate low excretions accurately.

As an additional check we have carried out further recovery tests in which known amounts of vitamin B_1 were added to urine. The recovery has always been virtually complete (Table 2).

Table 2. *Recovery of vitamin B_1 added to normal urines*

Specimen	Vitamin B_1 present μg. per ml.	Additional vitamin B_1 added μg. per ml.	Additional vitamin B_1 found μg. per ml.	Recovery %
Urine 1	0·118	0·16	0·15	94
	0·118	0·16	0·15	94
Urine 2	0·472	0·32	0·36	112
Urine 3	0·378	0·32	0·31	97
	0·408	0·32	0·287	90
Urine 4	0·0812	0·32	0·291	91

Since this note was written, a study by Slater [1941] has also appeared. His findings accord excellently with our own. He concludes: 'the thiochrome method of Wang & Harris can be satisfactorily applied to the determination of thiamin in milk and cereal products [and urine]....Criticisms by Hills [1939] of the Wang & Harris method of determining thiamin in urine appear to be unjustified....The recovery of added thiamin is quantitative in all cases.'

Summary

A further comparison has been made of the results obtained by the improved thiochrome method of Wang & Harris with those obtained by biological tests (bradycardia method). The agreement is good. On one specimen of urine the biological value was 110% of the chemical and on another 101%; in earlier tests the values were 100, 121, 100, 89, 128%; average for both series is 107%. Recovery tests confirm the reliability of the method.

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